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The effect of hypoxia on the metabolism of hyaluronan in cancer stem cells

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UNIVERSITE DE NAMUR

Faculté des sciences

Le métabolisme de l'acide hyaluronique dans les cellules souches cancéreuses en conditions hypoxiques

**Mémoire présenté pour l'obtention
du grade de master en biochimie et biologie moléculaire et cellulaire**

Stephanie FLETCHER

Janvier 2014



UNIVERSITY OF NAMUR

Faculty of Science

The effect of hypoxia on the metabolism of hyaluronan in cancer stem cells

**Master's Thesis presented for the obtention of a
Master's degree in biochemistry, cellular biology and molecular biology**

Stephanie FLETCHER

January 2014

Le métabolisme de l'acide hyaluronique dans les cellules souches cancéreuses en conditions hypoxiques

FLETCHER Stephanie

Résumé

L'implication des cellules souches cancéreuses (CSCs), aussi connues sous le nom de cellules initiatrices de tumeurs (TICs), dans l'initiation, la progression et la récurrence tumorale est un concept qui commence à prendre de l'ampleur. Les capacités d'auto-renouvellement et de division asymétrique des CSCs pourraient, en effet, expliquer l'hétérogénéité observée au sein des tumeurs. En outre, ces cellules seraient également responsables de la formation des métastases et de résistances aux chimiothérapies et à la radiothérapie (notamment via une entrée en quiescence). Le métabolisme de l'hyaluronan (HA) et un environnement pauvre en oxygène, l'hypoxie, sont deux facteurs impliqués dans la constitution d'une niche pour les CSCs et dans la progression tumorale. Ce travail est destiné à explorer la contribution de ces deux facteurs dans le développement de CSCs au sein de deux lignées cellulaires de cancer du sein, l'une (MCF-7) portant des récepteurs à estrogène et l'autre (MDA-MB-231) pas. Le modèle utilisé est celui des mammosphères (MSs), ou culture en conditions non adhérentes et en milieu défini, qui permet d'enrichir la population cellulaire en CSCs/TICs mais qui est également représentatif de l'hétérogénéité des tumeurs *in vivo*. L'étude a montré que l'hypoxie modifie la morphologie des MSs et le métabolisme de l'HA dans la lignée la plus agressive, MDA-MB-231, alors qu'elle accélère la croissance des MSs dans la lignée la moins agressive, MCF-7. L'interaction entre hypoxie et métabolisme de l'HA devrait être explorée plus en profondeur.

Mémoire de master en biochimie et biologie moléculaire et cellulaire

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Index Table

Index Table.....	2
I. Abbreviations	4
II. Introduction	6
A. Cancer stem cells and breast tissue.....	6
1. Generalities on stem cells and breast tissue	6
2. Breast structure	6
3. Stem cells	6
4. Maintaining the genome integrity in stem cells	7
5. Breast cancer and cancer stem cells	7
6. Cancer stem cell plasticity and heterogeneity	9
7. CSC isolation	9
B. Hyaluronan, its metabolism and its implication in cancer.....	10
1. Generalities on Hyaluronan	10
2. Hyaluronan metabolism	11
3. The association of hyaluronan with proteins	12
4. Biological functions of hyaluronan.....	12
5. Implication of hyaluronan in cancer	13
6. Implication of hyaluronan in stem cells	14
C. Hypoxia	15
1. Generalities on hypoxia	15
2. Causes of hypoxia and cellular responses.....	15
3. Taking advantage of hypoxia	16
4. The influence of hypoxia on hyaluronan metabolism.....	17
5. Hypoxia and cancer stem cells.....	17
6. Hypoxia, hyaluronan metabolism and cancer stem cells	19
III. Objectives.....	19
IV. Materials and Methods	21
A. Adherent cell lines	21
B. Mammosphere culture	21
C. Normoxic and hypoxic cultures.....	22
D. Mammosphere count	22
E. Immunofluorescence.....	22
F. Protein extraction and western blotting.....	22
1. Protein extraction and dosage	23
2. Sample preparation	23
3. Polyacrylamide gel preparation	23
4. Protein loading and electrophoresis	23
5. Semi-humid transfer.....	24
6. Protein detection	24
G. RNA extraction and Real-time reverse-transcription PCR.....	24
1. RNA extraction	25
2. Retro-transcription and quantitative PCR (RT-qPCR)	25
H. PH measurement.....	25
I. Hyaluronic acid dosage	26
1. Sample preparation	26
2. Dosage of hyaluronic acid.....	26
V. Results and discussion.....	28
A. Effect of hypoxia on the morphology of mammospheres	28

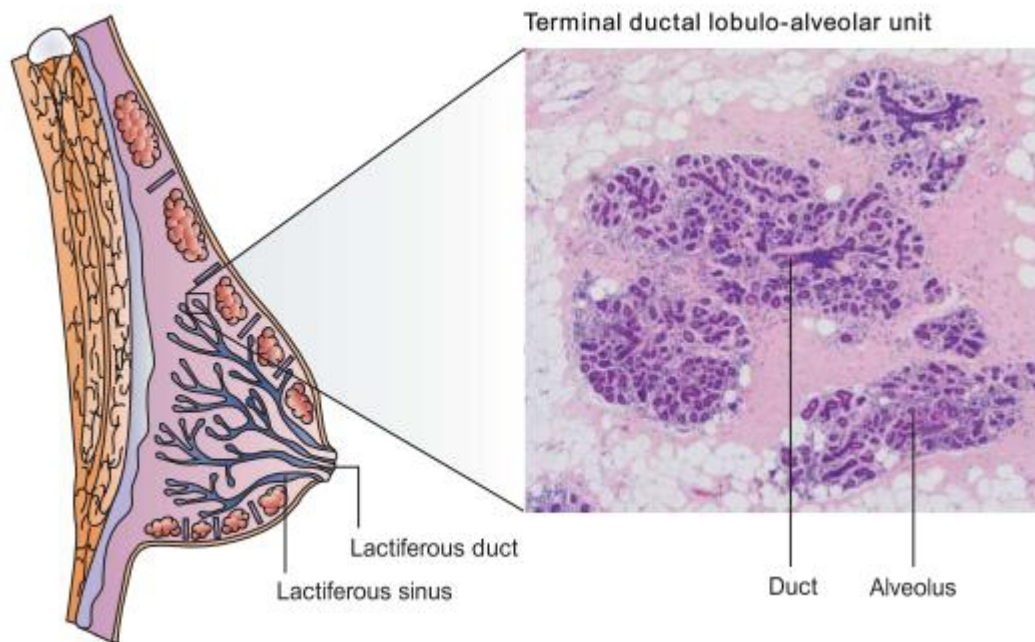
B.	Effect of different oxygen concentrations on mammosphere formation	29
C.	The effect of hypoxia on the pH of the mammosphere culture medium	30
D.	Effect of different oxygen concentrations on the metabolism of hyaluronan	31
1.	Effect of hypoxia on the production and secretion of hyaluronan	31
2.	CD44 expression in different oxygen conditions.....	32
3.	HAS2 expression in different oxygen conditions	33
4.	HAS3 expression in different oxygen conditions	34
5.	HYAL1 expression in different oxygen conditions	35
6.	HYAL2 expression in different oxygen conditions	35
VI.	Conclusion.....	36
VII.	Bibliography	41

I. Abbreviations

ABC transporter	ATP-binding cassette transporter
ALDH	Aldehyde dehydrogenase
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
BC	Breast Cancer
BMP	Bone morphogenetic protein
CD44s	Standard CD44 isoform
CD44v	Variable CD44 isoform
cDNA	complementary DNA
CpG	Cytosine phosphate guanine
CSC	Cancer stem cell
Da	Dalton
DLA	DIGE labelling buffer
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
ECM	Extra cellular matrix
EMT	Epithelial mesenchymal transition
FACS	Fluorescence assisted cell sorting assay
FBS	Fœtal bovine serum
GAG	Glycosaminoglycan
GLUT1	Glucose transporter 1
GTPase	Guanosine triphosphate hydrolase
HA	Hyaluronic acid or hyaluronan
HAS	Hyaluronic acid synthase
HAS1	Hyaluronic acid synthase 1
HAS2	Hyaluronic acid synthase 2
HAS3	Hyaluronic acid synthase 3
HIF	Hypoxia-inducible factor
HIF-1 α	Hypoxia-inducible factor 1 α
HIF-2 α	Hypoxia-inducible factor 2 α
HRE	Hypoxia response element
HRP	Horseradish peroxidase
HYAL	Hyaluronidase
HYAL1	Hyaluronidase 1
HYAL2	Hyaluronidase 2
HYAL3	Hyaluronidase 3
HYAL4	Hyaluronidase 4
iPSC	Induced pluripotent stem cells
LDH	Lactate dehydrogenase
MS	Mammosphere
PBS	Phosphate buffered saline

PHD	Prolyl hydroxylase domain
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PVDF	Polyvinylidene fluoride
pVHL	Von Hippel-Lindau tumour suppressor
RHAMM	Hyaluronan-mediated motility receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RTase	Reverse transcriptase
RT-qPCR	Real time quantitative polymerase chain reaction
TBS	Tris-buffered saline
TIC	Tumour initiating cells
TSG	Tumour suppressor gene
UDP	Uridine diphosphate

a)



b)

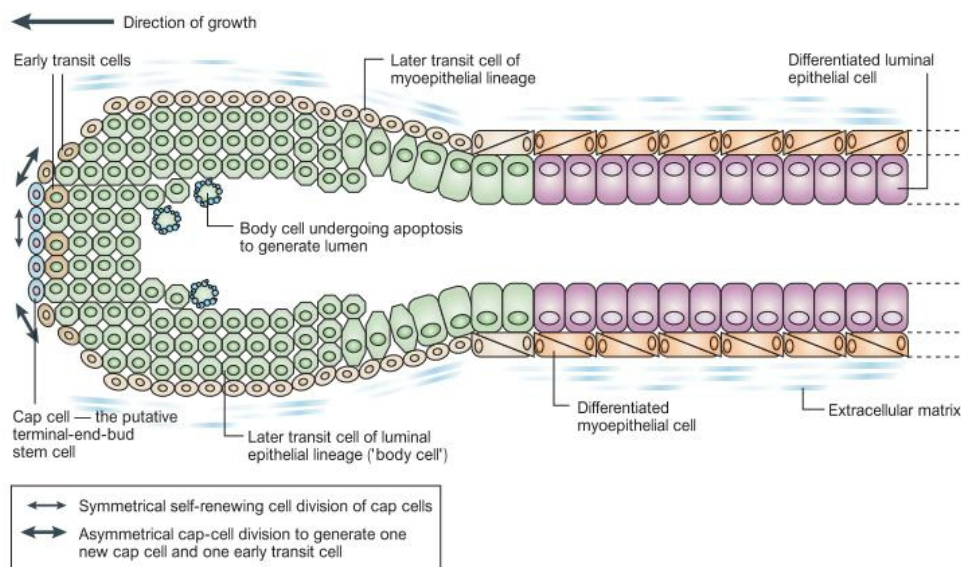


Figure 1. The human breast structure and the terminal end bud. a) On the left there is an illustrative representation of the epithelial structure of the human mammary gland. On the right there is photomicrograph of a haematoxylin- and eosin-stained cross-section through a terminal ductal lobulo-alveolar structure. b) Represents a terminal end-bud. The three cell-lines composing the gland are represented, as well as their localisation depending on their status of differentiation. The luminal cells or body cells create the alveoli by undergoing apoptosis. At the tip of the terminal end bud, are the stem cells that can undergo symmetric or asymmetric division.(Smalley & Ashworth, 2003)

II. Introduction

A. Cancer stem cells and breast tissue

1. Generalities on stem cells and breast tissue

As most epithelial tissues are under continual renewal, a stem cell component is necessary to ensure the plasticity of the tissue. Breast tissue, the focal point of this Master's Thesis, is an example of an organ where the presence of such a stem cell component is particularly important due to continual morphological changes throughout the female's life. To ensure the plasticity of these remodelling organs the body must coordinate and tightly regulate a hierarchy of at least three cell types, i.e. adult stem cells, amplifying cells, and post-mitotic differentiating or differentiated cells. The stem cells reside in a specific environment known as the stem cell niche, where not only the contact with other cells but also with the microenvironment is primordial for a balanced regulation of the cellular pathways. The niche in which these undifferentiated cells reside controls self-renewal and asymmetric division, thus allowing the maintenance of tissues and the ongoing development of organs (Chaffer et al., 2011; Dontu et al., 2003; Mackenzie, 2005; Spillane & Henderson, 2007).

The mammary gland is a unique organ in the sense that its development occurs primarily after birth, but also because it undergoes continual changes throughout the various stages of the woman's genital development. The epithelial cells composing the breast tissue respond to steroid hormones modifying the proliferative mechanisms to allow cyclical development of the organ (Charafe-Jauffret et al., 2008). To maintain the ongoing modifications to this particular tissue the stem cell component will ensure that the entire range of differentiated cells is present at all times by undergoing division and differentiation when a signal is received. One may surmise that a significant number of stem cells must be present in the breast tissue (Dontu et al., 2003; Ginestier et al., 2007a).

2. Breast structure

The repertoire of differentiated cells composing the breast tissue is made up of three different cell lineages that create the lobulo-alveolar structure of the mammary gland, where each lineage has its own specific functions. The alveolar epithelial cells, also known as body cells, synthesize the proteins that compose the milk produced during lactation. Myoepithelial cells form the basal layer of both the alveoli and the ducts; they are responsible for the expulsion of milk. The ductal epithelial cells line the lumen of the ducts (Dontu et al., 2003; Ginestier et al., 2007a). Together these three cell lines compose what is also known as the terminal end bud. At the tip of these buds are cap cells, which are thought to be the ductal stem cells responsible for maintaining the entire cellular repertoire and ensuring growth of the gland in response to external signals (Fig. 1) (Smalley & Ashworth, 2003).

3. Stem cells

Normal stem cells have both the ability to self renew and to undergo asymmetric division giving rise to a more differentiated progeny. The self-renewal of stem cells will provide a continuous pool of stem cells in the niche, from which the tissue can regenerate. Adult stem cells are multipotent, which means they have the ability to differentiate into various mature cells, but this capacity is usually limited to that of the specific tissue they occupy. When a stem cell undergoes asymmetric division the newly produced daughter cell can further divide and differentiate a certain number of times to eventually form the entire

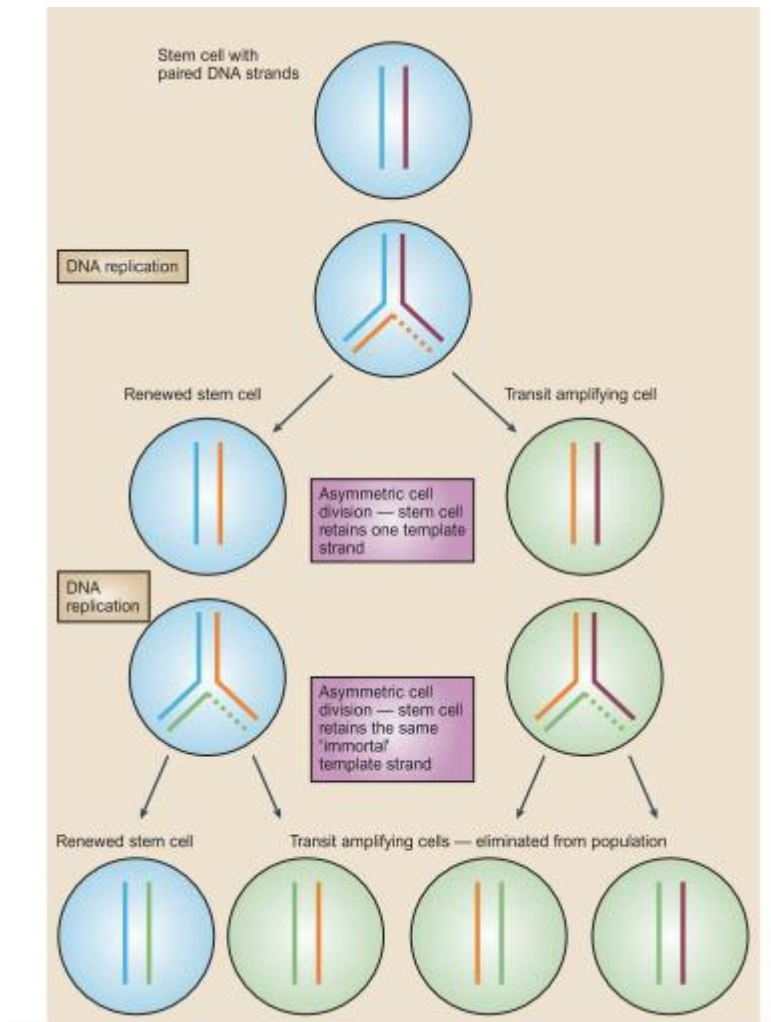


Figure 2. The immortal strand theory. After each asymmetric division a stem cell goes through, the template DNA strand (represented as the blue DNA strand) used for DNA replication prior to a division is always segregated to the stem cell. This phenomenon is specific to stem cells. The Renewed stem cell, will then go through the same process throughout its next asymmetric division. The immortal strand is passed down from one stem cell generation to the next. The daughter cell receives the newly synthesized DNA strand which may contain replicative associated errors that will later on be eliminated as these cells have a limited life span. This process aids in maintaining the integrity of the stem cell's genome, which is ever so crucial for a cell that is endowed with the role of regenerating the entire population of cells composing a certain tissue. (Smalley & Ashworth, 2003)

variety of cells contained in the mature tissue (Smalley & Ashworth, 2003). Stem cells are resistant to apoptosis and anoikis (survival in non-adherent conditions), have the ability to migrate, and their long life span ensures the development of mature tissue throughout the organism's entire lifetime (Spillane & Henderson, 2007). Although the stem cell can be active, such as undergoing asymmetric division and accomplishing self-renewal, another vital aspect of its life is the capacity to wait during long periods for a particular physiological sign (Smalley & Ashworth, 2003).

The regulation of these self-renewal pathways and the conservation of the stem cell pool are assured by transcription factors such as Notch, Oct4 and Nanog, as well as by signalling pathways transduced by Wnt/ β -catenin, Hedgehog, TGF- β and PTEN amongst others (F. Zhang et al., 2011). Experimentally it is possible to provoke the dedifferentiation of committed cells by inducing the expression of Oct4, Nanog, Sox2, KLF4, c-Myc, and miR-302. Oct4, Nanog, Sox2, KLF4, c-Myc are considered as the main stemness transcription factors. These particular proteins and micro RNAs are also known as induced pluripotent stem cell (iPSC) inducers (Mathieu et al., 2012).

4. Maintaining the genome integrity in stem cells

As mentioned previously, stem cells can be found in specific niches. These niches have been shown to offer protection from oxidative damage to the genome, which is an important precaution in cells with a long life span that also have the crucial role of regenerating tissues. Oxidative damage by reactive oxygen species (ROS) can be limited by low oxygen concentrations, otherwise known as hypoxia, and also by the presence of hyaluronic acid (HA) (Keith & Simon, 2007). HA is thought to have anti-oxidative properties by directly scavenging hydroxyl radicals and by chelating the ions necessary for Fenton's reactions, the reaction involved in producing the hydroxyl radicals (Darzynkiewicz & Balazs, 2012). Hypoxia and HA are both components of the stem cell niche, thus providing an ideal residence for stem cells (Keith & Simon, 2007).

Another way stem cells might ensure the integrity of their genome throughout their long life span was first described by Cairns in 1975. The hypothesis is that the dividing stem cells segregate the newly synthesized DNA strand to the amplifying cell while the template DNA strand is preserved in the stem cell. This is also known as the immortal strand hypothesis, as the very same DNA strand is maintained and passed from the initial stem cell to the renewed stem cell from one generation to the next. The preservation of one DNA strand after stem cell renewal is an attractive theory as it means that the stem cell genome would not integrate any replication associated errors (Fig. 2). Today there is research that backs this hypothesis (Mackenzie, 2005; Smalley & Ashworth, 2003).

5. Breast cancer and cancer stem cells

The exact origin of tumours is still a widely disputed topic. It is possible that they are the product of epigenetic and genetic modifications of differentiated cells but lately an increasingly popular view has been that they could arise from modifications to the stem cells' DNA. If these epigenetic or genetic alterations affect undifferentiated cells they could disturb the metabolic and the self-renewal pathways necessary for the homeostasis of the stem cells and consequently also that of the tissue they regenerate. Initial changes can give rise to pre-malignant sub-clones, i.e. cells that have enhanced proliferative activity when compared to normal cells. The body's natural defence mechanisms are able to keep these cells under control and inhibit the formation of malignant masses. These cells are considered as being pre-malignant. Some of the subclones may succumb to additional alterations or may receive

Somatic stem cell	Cancer stem cell
Self-renew, highly regulated	Self-renew, poorly regulated
Differentiate, produces mature tissue	Differentiate, produces tumour
Migrate to distant tissues	Metastasize to distant sites
Long lifespan	Long lifespan
Resistant to apoptosis	Resistant to apoptosis

Table 1. Comparison of somatic and cancer (Spillane & Henderson, 2007)

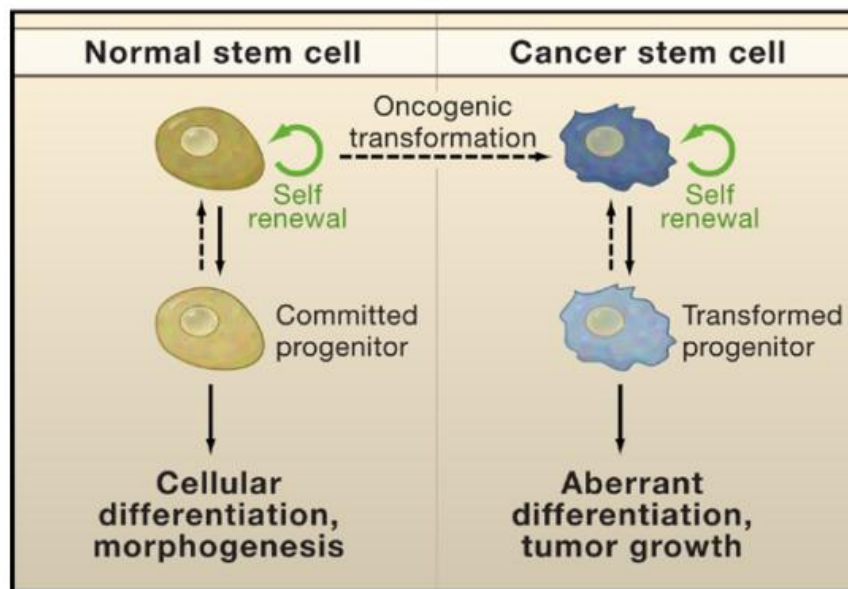


Figure 3. Normal adult stem cells compared to a cancer stem cell. Normal stem cells can self-renew to an extent that doesn't seem to be limited. The stem cells can also undergo asymmetric division giving rise to a more differentiated cell, a committed progenitor. Division in stem cells occurs at a slow rate but is highly regulated. These stem cells are important for guarantying the homeostasis of the tissue. Oncogenic transformations can perturb the regulation of the division pathways, thus leading to cancer stem cells which divide in an uncontrollable manner, which can lead to tumour formations. (Keith & Simon, 2007)

signals from a perturbed microenvironment. That may be sufficient to push the pre-malignant stem cells into gaining malignant functions, giving the cells the ability to overcome the body's control and develop into a malignant tumour mass (Bonnet & Dick, 1997; Grimshaw et al., 2008; Spillane & Henderson, 2007; Valent et al., 2012). Such aberrant stem cells are now known as cancer stem cells (CSCs) (Fig. 3, 4 and Table 1). There are indications that CSCs reside in niches that provide abnormal information to the stem cell, which could be a possible reason for the dysregulation of stemness pathways. The hypothesis that altered stem cells and cells presenting a stem-like phenotype are at the origin of tumours has given rise to the concept of the CSCs, otherwise also known as tumour initiating cells (TICs), defined as cells that have the capacity to produce observable invasive tumours or leukaemia when transplanted into experimental animal models (Fazilaty et al., 2013; Li et al., 2012; Valent et al., 2012). These xenografted CSCs have the unique ability of recreating heterogeneous tumours, an outcome that does not occur when injecting animals with the differentiated cellular population (Wiestler, Mumberg, & Haendler, 2006).

The aberrant communication between the cells and the niche may have numerous causes. Two of them are an aberrant response to hypoxia and an altered HA metabolism. They will be discussed later on. However, it is not yet known whether the miscommunication between the stem cell and its niche is due to the aberrant niche or whether the aberrant niche is a consequence of the apparition of CSCs (Gilbertson & Rich, 2007).

Stochastically speaking it is more probable that a stem or progenitor cell, with a long life span, would acquire one or two mutations dysregulating its self-renewal pathways than this phenomenon occurring in differentiated cells, usually having a short life span. As stem cells and CSCs are multipotent, any dysregulation in their differentiation pathways could explain the heterogeneity of tumours since the differentiation processes stemming from these cells would become uncontrollable (Fig. 5) (Charafe-Jauffret et al., 2008; Ginestier et al., 2007b; Spillane & Henderson, 2007).

Another possible reason for the generation of tumours could be a conversion of differentiated cells to cells presenting a stem-like phenotype, i.e. a dedifferentiation process (Chaffer et al., 2011; Dontu et al., 2003; Spillane & Henderson, 2007). Bearing in mind the immortal strand hypothesis, the conversion of a differentiated cell to a stem cell could be problematic if the genetic material passed onto the daughter cells had been subject to replication associated alterations. Due to the longevity of the stem cells the mutation in dedifferentiated cells would no longer be discarded as is the case in daughter cells. The maintenance of such mutations could give rise to a heterogeneous tumour after multiple divisions (Smalley & Ashworth, 2003). Which of the above CSC-linked theories best describes the reality of cancers is undecided yet.

CSCs have multiple characteristics that favour the development, the maintenance, and the recurrence of malignant tumours. One of these characteristics is the cell's resistance to chemo- and radiotherapy due to a high expression of ABC transporters, a good capacity to repair DNA damage, and a resistance to apoptosis (Table 1). Another characteristic is the ability of CSCs to enter quiescence. This is a state of dormancy that enables cells to survive in unfavourable environments, as is the case during cancer treatment, whilst also remaining ready to reengage in the normal cell cycle when the environment is more favourable (Charafe-Jauffret et al., 2008). Higher invasiveness can be associated with CSCs compared to the differentiated cell population. This could be due to an increased expression of proteins involved in infiltration such as vascular cell adhesion molecule (e.g. VCAM-1), integrins, chemokine receptors (e.g. CXCR4), and the HA receptor CD44 (Dontu et al., 2003; Mimeault & Batra, 2013a; Yu & Bian, 2009). When considering these characteristics favouring the development of a malignant tumour, it becomes obvious that therapies targeting these particular cells must be generated. However, due to conversion plasticity of CSCs to

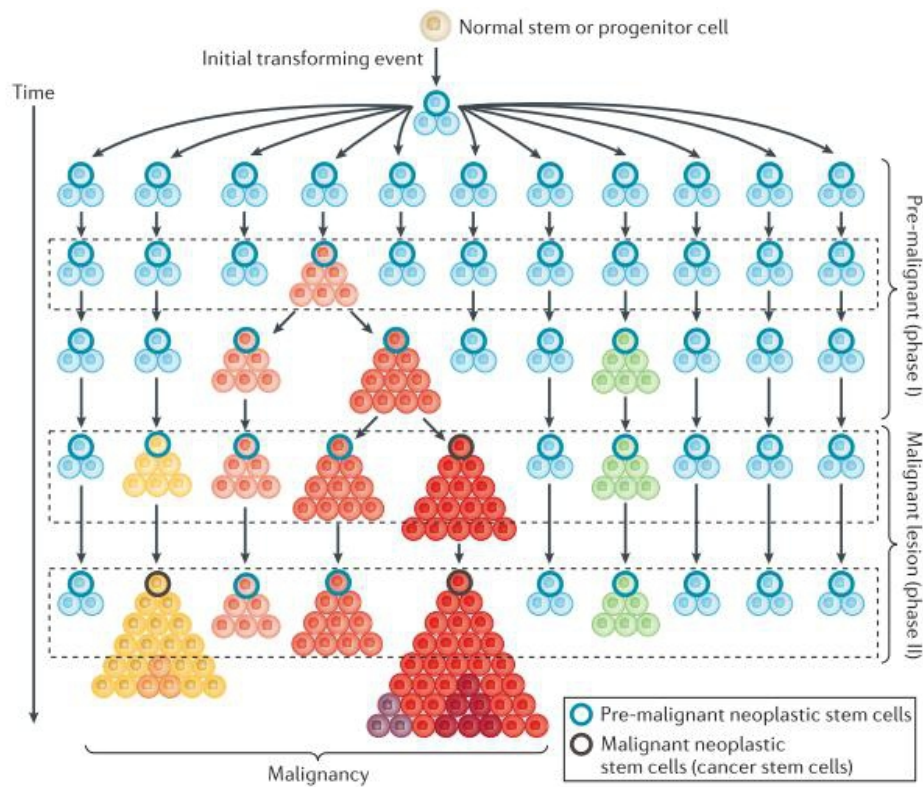


Figure 4. Model representing the evolution of cancer stem cells. Throughout the course of time a stem cell or progenitor cell may succumb to an epigenetic or genetic alteration. This alteration can provide progenitor cells with a stem-like phenotype. Proliferative abilities may be altered in a way that provides the cells with an advantage over normal cells. This Dysregulation of proliferative pathways may give rise to a persistent neoplastic clone. This initial clone, a pre-malignant stem (outlined in blue), can give rise to subclones that are naturally brought under control by the immune system or the surrounding tissue, preventing the development of malignancy. Additional alterations, genetic, epigenetic or changes in the microenvironment can provide these pre-malignant cells with the ability to overcome this first phase of cancer evolution and consequently allowing a malignancy to develop. This corresponds to the second phase of the cancer development. It is at this point that the neoplastic stem cells can be considered as cancer stem cells (outlined in black), as they have acquired the ability to initiate cancer. The pre-malignant stem cells may continue to proliferate continually generating other pre-malignant cells that may acquire the additional modifications that could lead to them developing into a malignant neoplastic cell or cancer stem cell. (Valent et al., 2012)

differentiated cells and vice versa, the differentiated population must still be targeted throughout treatment (Chaffer et al., 2011).

6. Cancer stem cell plasticity and heterogeneity

The degree of heterogeneity of tumours could be explained by multiple genetic and epigenetic alterations occurring in differentiated cells or by the various external signals that can be delivered to cancer cells depending on where they reside in the tumour, as different locations bear different microenvironments. But in order to reach the extent of heterogeneity seen in many cancers, many separate differentiated cells must succumb to these mutations, a phenomenon that is statistically improbable. A simpler explanation could be the CSC hypothesis. The heterogeneity could arise in response to altered self-renewal, proliferation and differentiation processes in CSCs generating a tumour composed of cells presenting phenotypical and functional differences. According to that second theory the proportion of CSCs composing the mass would significantly influence the degree of heterogeneity in the tumour, as the more CSCs there are the more possibilities of differentiation emanate, especially when taking into account the influence of the various microenvironments that can be observed in tumours (Magee, Piskounova, & Morrison, 2012; Pece et al., 2010).

The development of tumour heterogeneity is now often modelled as hierarchical, i.e. based on the CSC model, as opposed to the stochastic model, solely based on the differentiated population succumbing to a multitude of mutations. However, this hierarchical view is not entirely correct. There is evidently an array of functionally and phenotypically different cells composing the tumour mass. What is left out in this hierarchical model is the interconversional abilities of cells to change from one differentiated cell state to another. This can lead to problems while treating tumours as the targeted cells may be eradicated only to be replaced through the plastic abilities of CSCs or their daughter cells, leading to regrowth of the mass after the treatment. This means that even after targeting the initiators of tumours, the CSCs, the differentiated progeny could revert to a stem-like state if the adequate signals are received, thus enabling tumour regrowth after CSC targeting (Gupta, Chaffer, & Weinberg, 2009; Heddlestone et al., 2010; Pece et al., 2010). These issues bring to view just how crucial it is to target both differentiated and undifferentiated cells, and the necessity of developing treatments that will inhibit cellular plasticity.

7. CSC isolation

Charafe-Jauffrets *et al.*'s article in 2008 reviews the many ways of isolating and culturing CSCs *in vitro*, each coming with their own advantages and disadvantages (Charafe-Jauffret et al., 2008). CSCs can be isolated by the side population technique, which exploits the CSCs' ability to exclude Hoechst 33342 or Rhodamine 123 dyes. Fluorescent-Activated Cell sorting (FACS) can also discriminate CSCs from differentiated cells as breast CSCs typically have high and low expressions of the surface receptor proteins CD44 and CD24, respectively (CD44⁺/CD24⁻). Another method of isolating CSC is by the ALDEFLUOR assay which recognizes enzymatic Aldehyde Dehydrogenase 1 (ALDH1) activity.

The technique used in our laboratory for isolating and enriching CSCs is based on their resistance to anoikis. This ability to evade programmed cell death in non-adherent conditions enables the stem/progenitor cell population to be cultured into floating spheres *in vitro* that are made up of stem cells and their progeny. Mammary epithelial CSCs can form mammospheres (MS) when cultured in non-adherent conditions. If the adherent culture is adequately isolated one CSC or one early progenitor cell can give rise to one MS as they are the only ones able to survive in these non-adherent conditions. Within these MSs are cells that

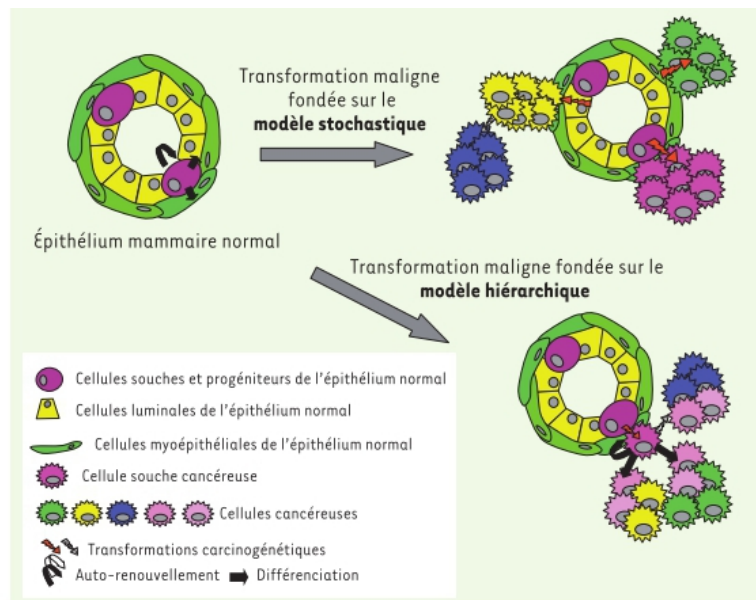


Figure 5. The stochastic and hierarchical models for tumour generation and heterogeneity. In the stochastic model, multiple differentiated cells of the mammary epithelium succumb to mutations that supply the cells with increased proliferative activities. Due to this increased ability to proliferate tumour formation composed of various differentiated cells can develop. In the hierarchical model it is a single stem cell that succumbs to alterations that can provide it with increased proliferative and self-renewal abilities. There will not only be generation of various differentiated cells but there is also a generation of other CSCs (possessing these same proliferative and self-renewal abilities), which can explain the heterogeneity of tumours. (Ginestier et al., 2007)

maintain both the ability to self-renew and to undergo asymmetric division. These progenitor cells can further undergo differentiation and lead to the development of MSs containing a wide range of diverse cells, that can even recreate functional mammary gland structures (Dontu et al., 2003; Ponti et al., 2005a). The monoclonal origin and refutation of cellular aggregation as an origin for MS formation was shown by analyzing secondary sphere formation with fluorescently transfected cells (Dontu et al., 2003). Ponti *et al.* showed that when injecting MCF-7 cells from MSs into immunodeficient mice, only 10^3 cells were sufficient to form tumours whereas a minimum of 10^6 cells from total adherent population were necessary to form a tumour in these mice (Ponti et al., 2005b). Even though this *in vitro* model does not replace a physiological environment, MSs are ethically favourable, cheaper and easier to study than the transplantation of stem cells into immunodeficient mice, all whilst maintaining the heterogeneity of *in vivo* tumours (Valent et al., 2012).

B. Hyaluronan, its metabolism and its implication in cancer

1. Generalities on Hyaluronan

HA is a simple disaccharide (GlcNAc β (1 \rightarrow 4)GlcUA β (1 \rightarrow 3)) repeated 2500 to 25000 times, forming a free glycosaminoglycan (GAG) polymer that plays a crucial role in the extracellular matrix (ECM) of cells. HA is unique as it differs from other GAGs in the sense that it is non sulphated, isn't attached to any core proteins, and its synthesis doesn't involve the Golgi pathway but is instead synthesised at the plasma membrane (Stern, 2004). This GAG is negatively charged at a physiological pH and is considered as a high molecular weight polymer as it can reach up to 10^4 kDa. The simplicity of this large polysaccharide does not merely reflect its intricate effects on the physiology and biology of vertebrates (Almond, 2007; Bastow et al., 2008; Hubbar et al., 2012a). HA forms a network around cells, known as the glycocalyx, which increases the expanse of their extracellular domain, intervenes in cell-cell interactions and can also act as a molecular sieve only allowing molecules of low molecular weight to pass (Almond, 2007; Bourguignon, Wong, Earle, & Chen, 2012a; Stern, 2004, 2005). The negative charge present on pseudo-random coil of HA polymers means that within aqueous solutions it will occupy a large space thus procuring it with the roles of hydrating tissues and ensuring that all spaces between neighbouring cells are occupied. It is additionally responsible for cell motility by providing the necessary extracellular space and interacting with the cytoskeleton (Fig. 6) (Almond, 2007; Stern, 2003, 2004).

Intriguingly HA appeared relatively late in the evolution of metazoan beings. HA is thought to play a role in the migration of foetal cells, allowing them to migrate more easily over significant distances, which does not occur in more primitive beings. It is also thought that HA may play a role in ensuring a physical separation between differentiated cells and undifferentiated cells. Thus a possible explanation for the late phylogenetic appearance of HA is that due to the fact that primitive organisms were mainly composed of multipotent cells, partitioning of the differentiated and undifferentiated cells was not necessary. The emergence of HA in later organisms could thus allow the separation of these different cells, allowing a more complex development (Stern, 2003).

The catabolism of HA leads to the formation of fragments of different sizes. These fragments are generated by hyaluronidases (HYALs), the enzymes responsible for HA degradation, and have very different and even contradicting effects on cellular responses depending on their size. Angiogenesis and inhibition of apoptosis are two examples of physiological effects that HA fragments can have on cells. An abundant amount of the fragmented form of HA can be a sign of malignancy (Almond, 2007; Stern, 2003, 2004).

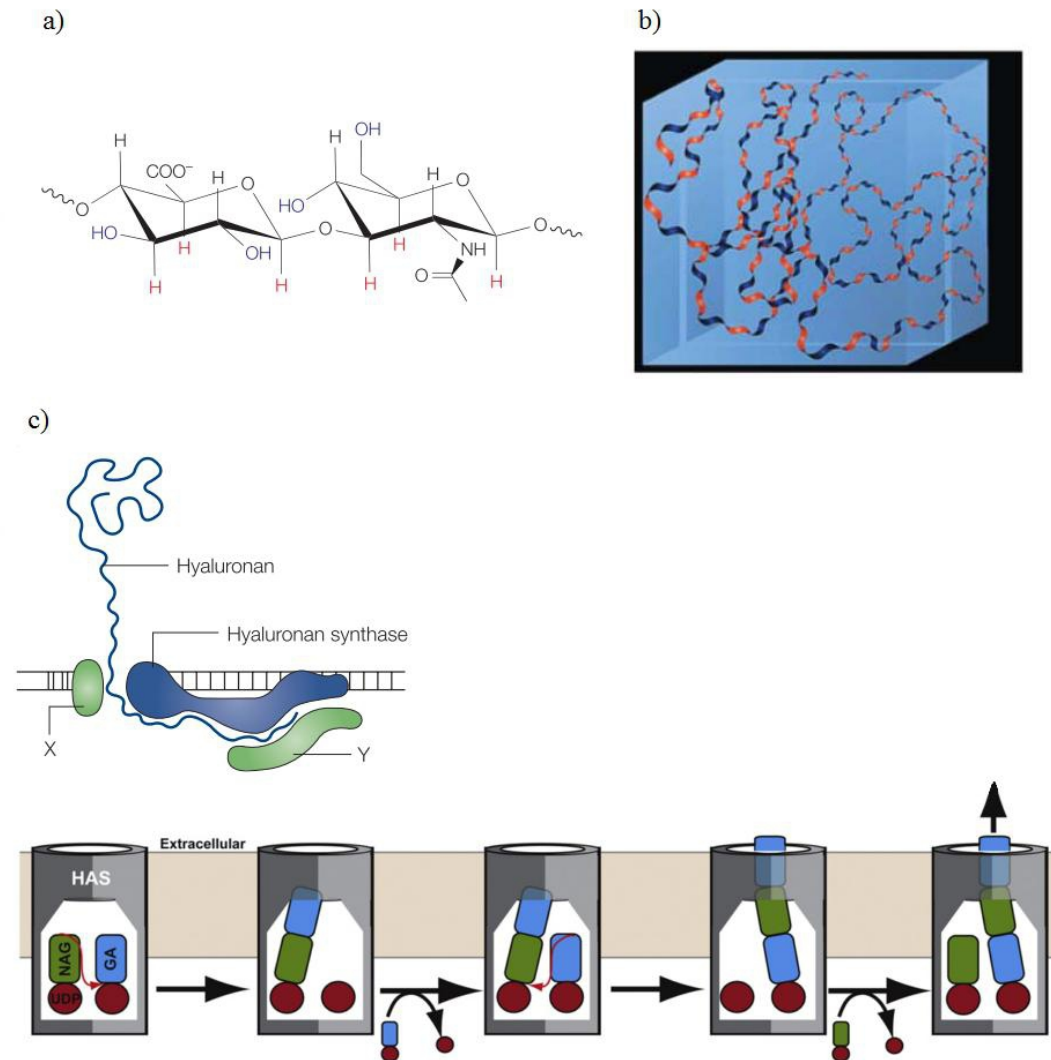


Figure 6. Structure of Hyaluronan, volume occupation by hyaluronic acid and hyaluronan synthesis. a) Representation of the disaccharide of glucuronic acid and N-acetylglucosamin, which is the basis of hyaluronan. These disaccharides are repeated many times in hyaluronan. The polymer composed of these disaccharides has charged and hydrophobic faces due to the presence of glucuronic acid's carboxyl groups on one side and hydrogen atoms on the other. b) The polymer occupies a large volume when it is present in a solution. The water molecules that get trapped within the polymer as well as the repulsion between carboxyl groups are responsible for the expansion of hyaluronan. Hyaluronan can execute internal pressure on physical structures providing the cells with routes that facilitate migration. c) Hyaluronan synthase enzymes are responsible for the synthesis of this polymer. At the inner membrane surface the monosaccharides are combined by a glycosyltransferase activity. Progressively the polymer is extruded to the outer plasma membrane where it can be retained by the enzyme or it can be released into the extracellular environment. X and Y represent putative regulatory proteins that aid the synthesis of hyaluronan. Adapted from (Hubbard, McNamara, Azumaya, Patel, & Zimmer, 2012; Toole, 2004)

Tumour development is one of the malignancies in which HA metabolism is known to be implicated, and these masses often present a high quantity of the fragmented HA form. Before reviewing how HA and its metabolism are involved in tumourigenesis it is important to go over the normal synthesis and degradation of HA, in order to understand the underlying problems that can drive tumour formation and malignant development.

2. Hyaluronan metabolism

a) Synthesis

In alternation, at the inner plasma membrane surface, one UDP-N-acetyl-D-glucosamine and one UDP-glucuronic-acid polymerise after losing their nucleoside diphosphate. This process is catalyzed by enzymes known as HA synthases, which exist in three isoforms (HAS1, HAS2 and HAS3) (Almond, 2007; Bastow et al., 2008; Stern, 2005). HAS proteins are integral membrane proteins embedded in the inner plasma membrane. During the elongation of HA the chain of repeated disaccharides is gradually extruded, but not secreted, towards the extracellular environment of the cell (Fig. 6) (Hubbard, McNamara, Azumaya, Patel, & Zimmer, 2012b; Rilla et al., 2013; Williams, Motiani, Giridhar, & Kasper, 2013).

HAS enzymes are present only in a very low concentration which leads researchers to believe that the main regulation of HA levels is done at the catabolic level (Stern, 2004). Not only is it important to regulate the concentration of HA in normal unaffected tissue but also during physical stress (tumourigenesis, septicaemia, wounds, burns, blood loss, etc.) as HA intervenes in the healing processes of such lesions. Rapid increases in HA levels occur at affected sites and must thereafter be decreased with the help of HYALs (Stern, 2008a).

b) Degradation

The daily turnover of HA and the degradation of HA after the wound healing process are both carried out by HYALs. They can fragment HA into polymers of different sizes ranging from 20 kDa fragments to tetrasaccharides. With the help of this hyaluronidase activity, the daily turnover rate of HA in vertebrates is estimated to be of 33 %, which is considered to be particularly high. The turnover is even greater in malignant tissues such as tumours where one can find a high concentration of fragmented HA (Stern, 2008a). In mammals the HYALs responsible for the degradation of HA are endo- β -acetylhexoaminidases; they fragment HA by hydrolysis. There are six hyaluronidase-like genes (*HYAL1*, *HYAL2*, *HYAL3*, *HYAL4*, *PHYAL1* and *SPAM1*) present in the human genome, but it is *HYAL1* and *HYAL2* that are responsible for the majority of HA catabolism (Bastow et al., 2008; Stern, 2004). Both of these HYALs are acid active, and both are present in two isoforms. *HYAL2* has a glycosylphosphatidylinositol (GPI) link that anchors it to the plasma membrane. *HYAL1* has a molecular weight of 57 kDa or 45 kDa depending on whether it has been processed by an endoprotease reaction (Stern, 2004, 2005). It can be found in the extracellular space as well as within the cells. There is some conflicting information on the subcellular localisation of *HYAL1*, although there are indications leading researchers to believe that it may be located and effectuate its function in early lysosomes (Andre et al., 2011; Gasingirwa et al., 2010; Stern, 2003, 2004, 2005). Stern even suggests the possible existence of an organelle dedicated to the catabolism of HA, first described by Mian in 1986, known as the hyaluronosome (Mian, 1986; Stern, 2003).

HYAL2 fragments HA of high-molecular mass into polymers of approximately 20 kDa. *HYAL1* on the other hand doesn't have size specific activity and can consequently

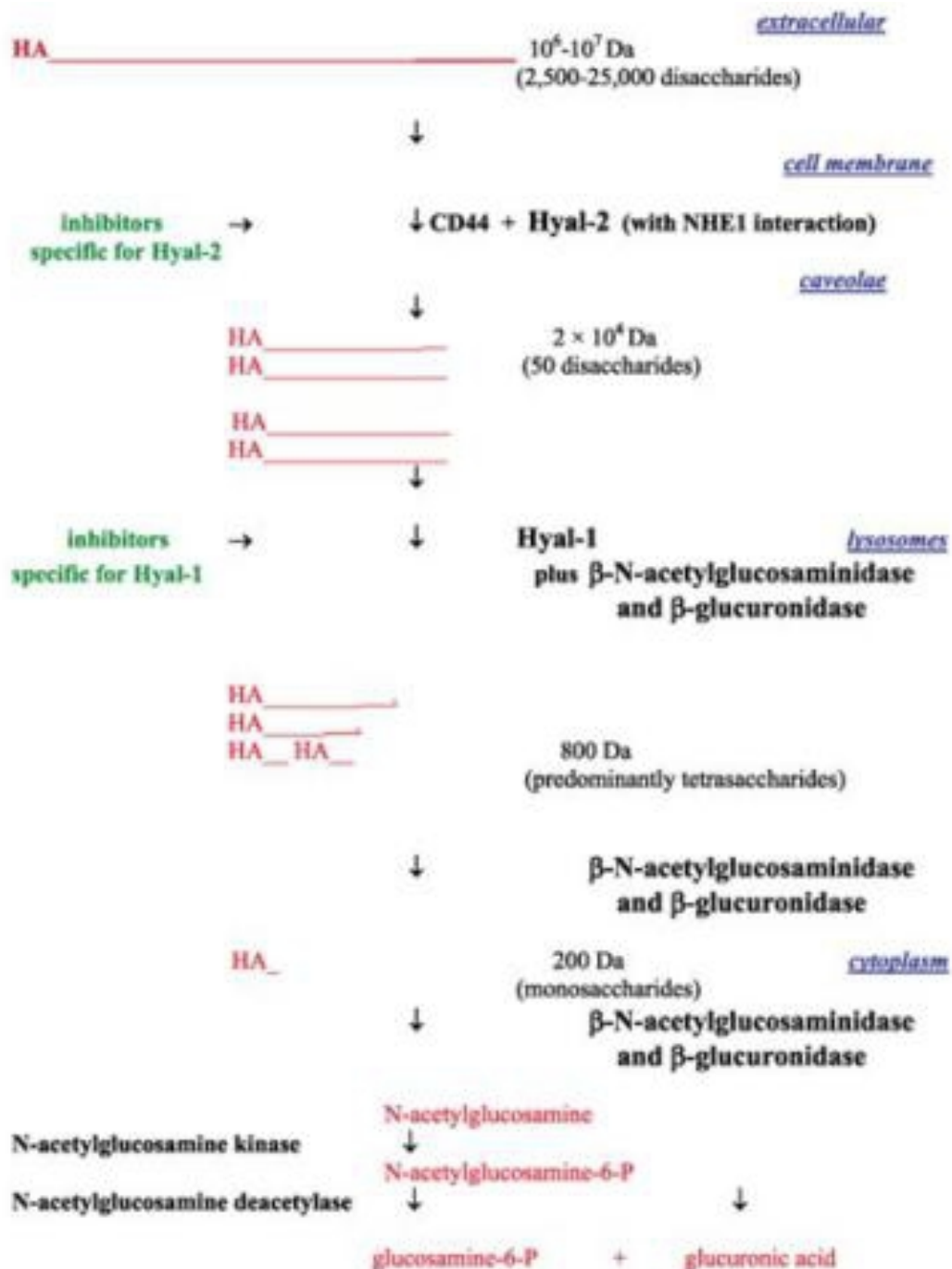


Figure 7. Hyaluronan degradation. This is the widely accepted cascade scheme for hyaluronan catabolism. It starts with the high molecular weight polymer which is internalised at microdomains known as caveola and degraded by HYAL-2 into fragments of 50 disaccharides. Next HA is fragmented into smaller fragments by HYAL-1, N-acetylglucosaminidases and glucorinidases. (Stern, 2004)

degrade HA of all sizes into smaller fragments such as tetrasaccharides and disaccharides (Stern, 2004). The catabolism of HA initiates at specific sites on the cell membrane known as lipid rafts. At these microdomains HA is taken up into the cells after recognition by CD44. CD44, a transmembrane cell surface receptor and also an adhesion protein, can interact with HYAL2 during the catabolism of HA. At these particular microdomains a HA-CD44-HYAL2 complex will initiate the cleavage of HA into smaller fragments. As HYAL2 is acid-active, a low pH is ensured by a Na^+/H^+ exchanger. After internalisation the fragments will follow the endocytic membrane transport pathway by first being delivered to endosomes and then probably to lysosomes. Supposedly it is within the lysosomes that HYAL1 will further degrade the HA fragments into limit fragments with the help of three other enzymes, notably β -exoglycosidases, β -glucuronidases and β -N-acetylglucosaminidases (Fig. 7) (Bastow et al., 2008; Ponta, Sherman, & Herrlich, 2003; Stern, 2004, 2008a; Williams et al., 2013).

Another means of HA degradation is by ROS. High molecular weight (HMW) HA has ROS-scavenging abilities by which it directly interacts with the oxidants, which leads to the breakdown of the polymer of HA (Darzynkiewicz & Balazs, 2012).

When an increase in HA is necessary, as is the case during an important physiological stress, HYAL inhibitors are released in response, e.g. to IL-1, which will rapidly inhibit the body from degrading HA. In normal healthy tissues the presence of HYAL inhibitors is very important as they help maintain the extracellular matrix (ECM) by stopping HA degradation when it is not necessary (Stern, 2004, 2008a).

3. The association of hyaluronan with proteins

The binding of HA to CD44 doesn't solely play a role in the uptake of the polymer for metabolism, it is also highly involved in signal transduction which is involved in cellular differentiation, proliferation, survival and motility. RHAMM is another receptor capable of transducing signals involved in cellular processes such as proliferation, survival and motility after having bound HA. The involvement of hyaladherins, in particular CD44, is relevant to this particular study as the mentioned cellular processes are implicated in stemness and tumourigenesis (Bourguignon et al., 2008; Bourguignon et al., 2012a; Bourguignon et al., 2012b; Hiraga, Ito, & Nakamura, 2013; Toole, 2004).

CD44 is made up of a single chain which presents an ecto-, a transmembrane-, and a cytoplasmic domain. Multiple isoforms of CD44, one standard form and a number of alternative forms, exist as this adhesion glycoprotein can undergo various combinations of splicing during the transcription of its gene (Olsson et al., 2011; Thorne, Legg, & Isacke, 2004; Toole, 2004). HA binds to the extracellular domain which can activate the receptor. In response to HA the cytoplasmic domain can interact with an array of proteins such as tyrosine kinases, phosphatidylinositol 3-kinase (PI3K), PKC, RAS, ERBB and Nanog. It is via these signalling pathways that HA bound to CD44 is involved in tumour development, maintenance and metastasis (Bourguignon et al., 2008; Toole, 2004).

4. Biological functions of hyaluronan

Depending on the size and molecular weight of the HA fragments different physiological effects can be induced on the organism. As previously mentioned HMW HA has a role of filling the voids between cells and for hydrating tissues. This HMW polymer also has anti-angiogenic, anti-inflammatory and immunosuppressive effects on the organism. The intermediate sized HA fragments, weighing between 20 kDa and 6 kDa, promote inflammation by stimulating the production of pro-inflammatory cytokines, and enhancing angiogenesis, the immune response and the endothelial recognition of injury. The

HA fragment	Physiological effect of HA fragment
>100 kDa (extracellular high-molecular weight polymer)	Space filling
	Hydrating
	Anti-angiogenic
	Anti-inflammatory
	Immunosuppressive
20 kDa (intermediate or low molecular weight fragment)	Angiogenic
	Immunostimulatory
	Inflammatory
	Endothelial injury recognition
	MMP expression
Small oligomers: tetrasaccharides	Induction of heat-shock proteins
	Anti-apoptotic
	Anchorage independent growth

Table 2. Size dependent physiological effects of HA on the organism (Stern, 2004)

transcription of matrix metalloproteases (MMPs) can also be induced by these intermediate size fragments of HA. The smaller HA fragments known as oligosaccharides having a chain of 3 to 10 disaccharides can stimulate anchorage independent growth of tumour cells. The expression of heat-shock proteins can be activated by tetrasaccharides, which can also have antiapoptotic effects. A summary of these physiological effects is available in Table 2. It's quite astonishing to see how some of these effects are completely contradicting. This just shows the complexity of HA and indicates that its regulation must be closely monitored in order to maintain not only cellular but also organismal homeostasis. Defects in the metabolism of HA have been seen to lead to malignancies as is the case in tumour development (Stern, 2003, 2004).

5. Implication of hyaluronan in cancer

The tumour cell's inherently instable genome means that it frequently succumbs to change, which can make it difficult to truly understand the underlying mechanisms that lead to the development of such malignancies in the first place (Stern, 2005, 2008a). It has in fact been shown that HA and its metabolic partners such as HYALs, HYAL inhibitors and CD44 play an important role in tumour progression (Hiraga et al., 2013; Stern, 2004, 2008a; Sugahara et al., 2003).

Malignant tumours present higher concentrations of HA than normal tissues and have a considerable influence on the sheer mass of the tumour (Stern, 2005, 2008b). This can either be due to a high production of HA by the tumour cells themselves or to the synthesis of HA by the surrounding cells making up the microenvironment. The area surrounding the tumour known as the peritumour stroma has certain similarities to embryonic stroma. How this "embryonic environment" develops is not yet clear, but it is speculated that the tumour's paracrine signalling intervenes in the process (Stern, 2005). Abnormal HA metabolism is one of many components of the peritumour stroma which promotes tumour cell proliferation, invasion and metastasis (Hiraga et al., 2013; Stern, 2005). In ovarian and breast cancer (BC) there is an inverse relationship between the levels of HA in the tumour stroma and the rate of patient survival. It has also become evident that the visualisation of the areas presenting an increased amount of HA by sonography can give a clearer idea on the shape and size of the tumour than a mammography (Toole, 2004).

Overexpression of HAS proteins can be observed in various cancers, such as fibrosarcoma, prostate carcinoma and mammary carcinoma. A recent paper notes that HAS2 is overexpressed in breast CSCs; this will be detailed in the point addressing the impact of HA on normal stem cells and CSCs. In metastatic colon cancer there is an overexpression of HAS3 and an increased amount of HA. The up-regulation of this protein facilitates anchorage independent growth and promotes growth and progression of colon carcinoma (Bullard et al., 2003; Okuda et al., 2012).

HYAL1 and HYAL2 are both contributors to cancer progression in their own way. HYAL1 is considered to be the product of a tumour suppressor gene (TSG). Cancers have the ability to eradicate any activity that is harmful to the tumour itself (Stern, 2004). In many cancers such as breast cancer the gene can either be deleted or silenced epigenetically through a hypermethylation of CpG islands or through a silencing at the RNA level (Stern, 2005). Inhibition of HYAL1 entails an accumulation of oligosaccharides which promote malignant transformation and progression by the stimulation of mechanisms such as angiogenesis (Stern, 2008a). HYAL2 is considered to be an oncogene as well as a TSG. In some instances its overexpression is accompanied by an acceleration of tumour progression. By increasing the rate of apoptosis the gene coding for this enzyme can also be considered as a TSG (Stern, 2004, 2005).

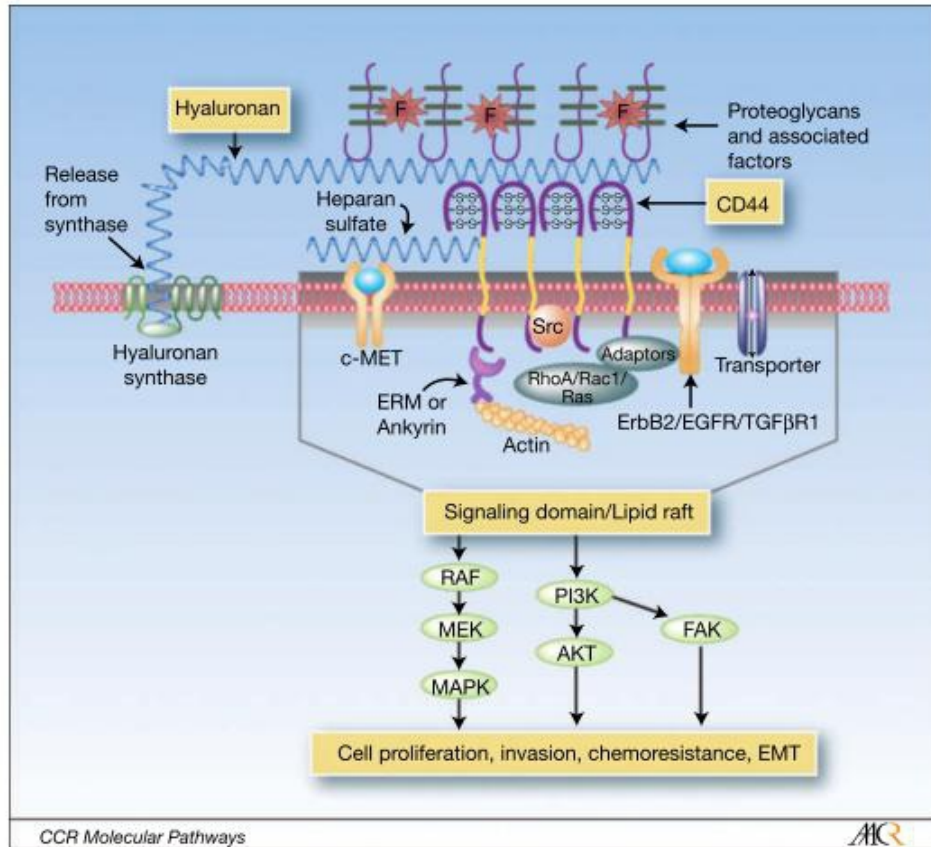


Figure 8. The regulation of signalling cascades by the CD44-hyaluronan interaction. The hyaluronan synthesised by hyaluronan synthases is extruded to the plasma membrane. There the polymer can interact with proteoglycans and very importantly CD44, the main HA receptor. During HA's interaction with CD44 there is induction and/or stabilisation of its signalling domains located in the plasma membrane. These domains hold receptor tyrosine kinases such as ErbB2 and EGFR and non-receptor kinases such as the Src family. These enzymes are drivers of oncogenic pathways via MAP kinase and PI3 kinase/Akt cell that stimulate proliferation and survival pathways as well as the expression of transporters that provide the cells with drug resistance properties and contribute to the acquisition of malignant properties. Via the interaction with adaptor proteins these signalling pathways can be regulated. This schematic view of the effects of the interaction between CD44 and HA elucidate how autonomous activities can be induced and maintained in tumour cells (Toole, 2009)

HYAL inhibitors are important for maintaining a functional and healthy ECM, as they can regulate the rate of metabolism of HA. It has become apparent that cancer patients have different classes of HYAL inhibitors compared to healthy patients. In these patients the presence of constitutively active forms of inhibitors are common. By inhibiting HYAL1 an accumulation of intermediate-sized HA fragments can stimulate angiogenesis which is a known contributor to cancer progression (Stern, 2005, 2008a).

Intermediate sized fragments of HA can also induce the cleavage of CD44 from the cell surface. Cell growth can thus become adhesion independent as the cleavage of CD44 by metalloproteases can mediate motility and invasion of cancer cells. Elevated levels of CD44 cleavage have been seen in colon, ovarian, non-small cell lung, and breast cancers (Sugahara et al., 2003; Thorne et al., 2004).

As previously mentioned the interaction between HA and surface receptors such as CD44 has an impact on cell survival, proliferation and motility. Cellular responses engaging PI3K, protein kinase C (PKC), RAS and other such enzymes known to be highly implicated in tumour development are activated. Any disruptions in these pathways could be obvious contributors to tumour development and progression (Fig. 8) (Toole, 2004).

The elevated amounts of HA present in cancerous tissue can also protect the tumour cells from the immune system due to the formation of a protective barrier (Sugahara et al., 2003).

6. Implication of hyaluronan in stem cells

An indicator that HA is vital for stem cells is the fact these cells have a high expression of the main HA receptor, CD44. This glycoprotein when used as a stem cell marker can discriminate the undifferentiated population from the differentiated one. Through this receptor many different signals can be transduced that are involved in processes such as the alteration or maintenance of the cell's differentiation status, inducing migration but also in processes linked to tumourigenesis.

Certain transcription factors associated with stemness can be activated by HA. This is the case for Nanog, Oct4 and Sox2 which are all stem cell markers. When HA binds to CD44 it can interact and activate PKC which in turn phosphorylates Nanog. Under this activated state nuclear translocation of Nanog is possible, thus allowing it to induce the transcription of genes that are involved in reducing apoptosis and chemosensitivity, which are some of the characteristics of CSCs. In another model HA binds to CD44v3 and induces the production of miR-302 through the CD44-HA induced nuclear translocation of a transcription factor complex made up of Oct4, Sox2 and Nanog. miR-302 induces the expression of genes involved in stemness (Fig. 9) (Bourguignon et al., 2008).

HA's ability to create an embryonic-like environment is of considerable importance for stem cells as it will allow them to maintain their genomic integrity. For CSCs this is even more of an advantage as it means that the cells could be protected from the different treatments aiming to damage the DNA in order to rid the patient of the aberrant cells forming the tumour mass (Charafe-Jauffret et al., 2008; Darzynkiewicz & Balazs, 2012; Keith & Simon, 2007; Stern, 2005). This protective environment means additionally that any mutations that may have already been acquired, by the CSC or by daughter cells reverting back to a stem-like phenotype, can be maintained and, through asymmetric cell divisions these mutations can be propagated (Charafe-Jauffret et al., 2008; Spillane & Henderson, 2007).

It has been reported that the cleavage of the ectodomain of CD44 is an important step for cell migration in a HA based ECM. Shedding this glycoprotein at the rear of the cell is linked to detachment from the matrix. The front of the cell, the side leading the migration,

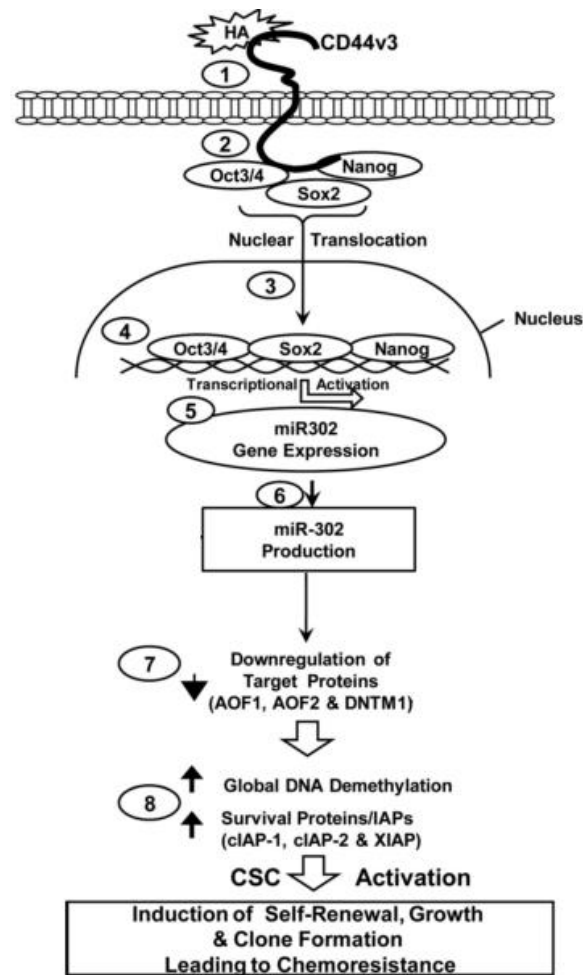


Figure 9. A model for the regulation of cancer stem cell functions via CD44-HA mediated Oct4-Sox2-Nanog signalling induced miR-302 production. In step 1, HA binds to CD44 which interacts with the Oct4-Sox2-Nanog complex (step 2). In step 3, the transcription factor complex can translocate to the nucleus, and bind to the promoter of the miR-302 cluster (step 4). The binding of this complex induces the transcription of miR-302 (step 5) and the production of mature miR-302 (step 6). miR-302 downregulates protein involved in epigenetic regulation (step 7 and 8) and up-regulates survival proteins (step 8) which increases selfrenewal, clonal formation and contributes to chemoresistance. Adapted from (Bourguignon, Wong, Earle, & Chen, 2012)

will have a high expression of CD44, allowing the cell to latch on to the ECM. Thus this repetition of attaching and detaching accompanied by a cytoskeletal rearrangement at both poles of the cell will allow the cell to move forward (Nagano & Saya, 2004).

A recent publication has revealed that a high expression of HAS2 is vital in breast CSCs for bone metastasis (Okuda et al., 2012). The high expression of this enzyme appears to be linked to better adhesion to endothelial cells and increased ability to transmigrate through an endothelial monolayer. The increased production of HA by HAS2 increases the CSC's interaction with stromal cells in the bone, thus contributing to increased proliferation. The authors point out the fact that HAS2 is an essential factor in providing CSC with the ability to metastasize. Their findings show that metastatic CSCs have an elevated expression of HAS2 and that HA is an important factor in the interaction with surrounding cells such as tumour associated macrophages. This interaction can stimulate the secretion of growth factors that in turn stimulate the growth of CSC (Okuda et al., 2012).

C. Hypoxia

1. Generalities on hypoxia

Once a tumour has grown to a certain size the oxygen delivery to the cells composing the mass is no longer adequate, leading to hypoxic zones. Hypoxia is defined as any oxygen tension too low to allow normal cells and organs to function in their typical way. These low concentrations of oxygen don't provide a favourable microenvironment for most cells, entailing the appearance of necrotic zones. These zones can be observed in tissue areas that are situated approximately 70 μm -180 μm away from a blood vessel. Although hypoxia is considered to be toxic for most cells, some cells, such as stem cells and cancer cells have or can acquire mechanisms to survive and proliferate in these conditions. This low concentration of oxygen is thought to favour and allow the maintenance of a stem cell phenotype as well as contribute to altered HA metabolism via the intermediate of compounds such as Hypoxia-inducible factors (HIFs) and lactate (Keith & Simon, 2007; Y. Kim, Lin, Zeltermann, & Yun, 2009; Krishnamachary et al., 2012; Stern, 2008a; Vaupel, Höckel, & Mayer, 2007)

2. Causes of hypoxia and cellular responses

Hypoxia is a localised condition that occurs in solid tumours due to decreased oxygen availability. The low O_2 partial pressure is a result of poor vascularisation and over-consumption of oxygen due to the high proliferative activity of the tumour mass. The hypoxia can also be caused by anaemia induced by the therapies aiming to treat cancer (Keith & Simon, 2007; Vaupel et al., 2007). The key response elements inducing the adaptive changes in hypoxic conditions are HIFs. One of the most studied responses that is induced through this pathway is the VEGF activated neoangiogenesis (Harris, 2002). These mediators of the response to low oxygen levels are activated when physically the cells are localised too far away from an oxygen source. It's not only the increased distance between the cells and blood vessels that leads to hypoxia but also the fact that the rapidly induced angiogenesis produces poor neovasculature. These newly synthesized blood vessels are leaky and unorganised (Liao & Johnson, 2007). Hypoxia is considered to be toxic for differentiated normal and cancer cells and usually induces cell death by activating p53 or up-regulating NIX and NIP3 (mediators of cytochrome c independent cell death). But cancer cells are able to adapt to this particular form of stress. Cancer cells have the ability to not only survive in this oxygen deprived microenvironment but also to take advantage of it. Increased malignancy, poor

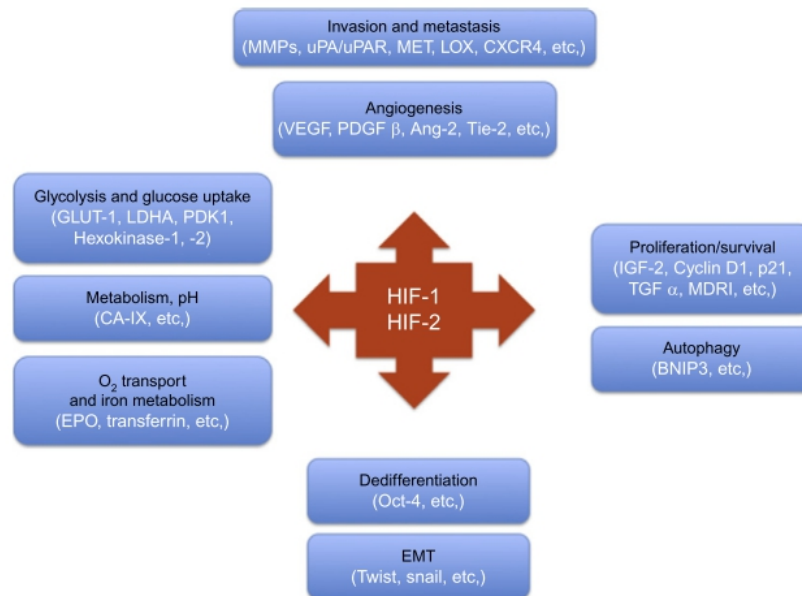


Figure 10. A summary of some of the genes activated by HIF-1 α and HIF-2 α . The genes activated by these HIFs are involved in processes such as: dedifferentiation, EMT, proliferation, survival, angiogenesis, invasion, metastasis, metabolism and O₂ transport. (Cuvillier et al., 2013)

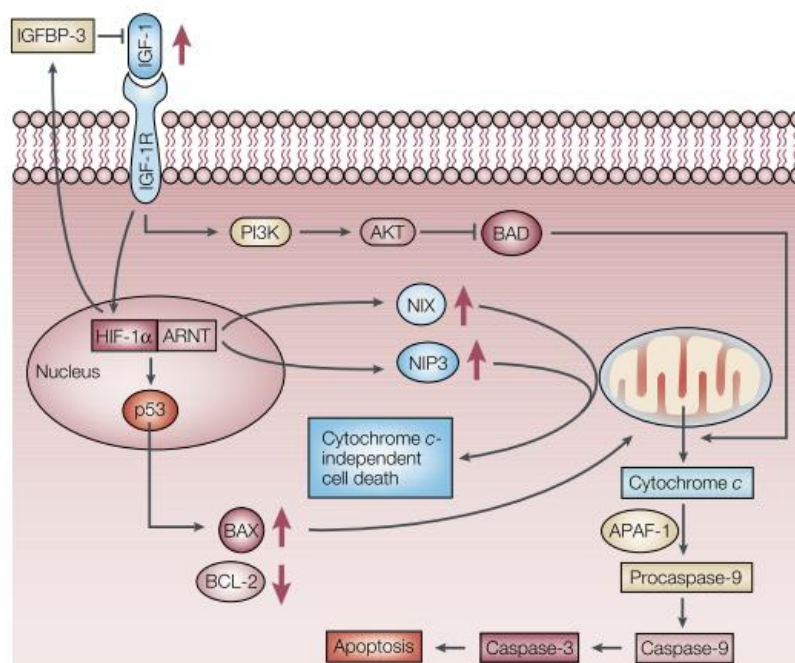


Figure 11. Regulation of cell-death pathways by hypoxia. HIF-1 α activates the transcription of many pro-apoptotic genes, induces cytochrome c independent cell death and activates p53 an inducer of apoptosis. HIF-1 α also induces the transcription of a pro-apoptotic protein IGF-binding protein 3 (IGFBP-3). IGFBP-3 inhibits IGF-1R signalling. This signalling first activates a cascade which inhibits BAD, a pro-apoptotic protein. So by inhibiting IGF-1R this inhibition is lifted and apoptosis can be induced. (Harris, 2002)

patient outcome and resistance to chemo- and radiotherapy are accompanied by tumour hypoxia (Harris, 2002; Keith, Johnson, & Simon, 2012).

HIFs are heterodimeric complexes that comprise an O₂ regulated bHLH-PAS α -subunit and an aryl hydrocarbon nuclear translocator (ARNT) β -subunit (Al Okail, 2010; Harris, 2002; Keith et al., 2012). The α -subunit exists in 3 isoforms, HIF-1 α , HIF-2 α and HIF-3 α . Information on the effects, the localisation and the induction of HIF-3 α are scarce. HIF-2's localisation is thought to be developmental stage- and tissue specific whereas HIF-1 is ubiquitously expressed in all cells (Chiavarina et al., 2012). In the presence of O₂ the α -subunits of HIF undergo HIF-specific prolyl-hydroxylase (PHD) modification which leads to rapid proteasomal degradation. This degradation is mediated by the Von Hippel Lindau (pVHL) tumour suppressor protein which can only recognize HIF in its hydroxylated state. Redox-dependent proteolytic stabilisation of the α -subunit prevents proteasomal degradation and enables translocation to the nucleus where it can interact with the oxygen independent HIF β -subunit (Chilov et al., 1999). Once in the nucleus the α - and β -subunits form a complex that can bind to hypoxia-response elements (HRE) and induce the transcription of multiple genes. The two highly homologous isoforms HIF-1 α and HIF-2 α are activated by different oxygen tensions and share many but not all HRE binding sites. This means that the two different HIFs activate some common genes while other gene inductions are isoform-specific (Keith & Simon, 2007; Li et al., 2009). Severe hypoxia is considered as an oxygen level of 1 % or below, and moderate hypoxia ranges from above 1 % to 5 % oxygen. Severe hypoxia is necessary to activate HIF-1 α whereas moderate hypoxia is sufficient to stabilise HIF-2 α and allow its nuclear translocation (Li et al., 2009). The genes activated are involved in oxygen transport, glucose uptake, stemness, stress response pathways, cell adhesion, migration, and immortalisation (Fig. 10). One of the HIF-1 α induced cellular responses is the induction of apoptosis which decreases the proliferation rate of the tumour by stimulating pro-apoptotic proteins such as NIX, NIP3 and p53 (Fig. 11) (Cuvillier et al., 2013; Harris, 2002).

3. Taking advantage of hypoxia

The ability of cancer cells to adapt to a multitude of environments and to bypass situations in which they are receiving signals to undergo apoptosis is extraordinary (Fig. 12). They can manage to escape treatments causing damage to their DNA and to survive in environments deprived of nutrients and oxygen (Harris, 2002).

Many cancer cells overcome hypoxia-induced apoptosis by overexpressing anti-apoptotic genes such as *IAP2* or by modulating mediators of the apoptotic pathway. Hypoxia can increase the activity of PI3K which intervenes in pathways that stimulate proliferation and inhibit apoptosis. HIF-1 α can increase the expression of RAS, a potent oncoprotein and GTPase responsible for the activation of proteins involved in growth, differentiation and survival. Hypoxia participates in immortalising cancer cells. This is possible through the enhanced activity of telomerase enzymes enabling the cell to overcome its normally restricted replicative capacity. Many modifications to the DNA of oncogenic cells are not only provoked but also maintained by hypoxia. DNA repair in many of these transformed cells is impaired when they are cultured in low pH and low oxygen environments. Gene amplification is induced and fragile sites on chromosomes are more likely to succumb to DNA breaks. This increases genetic instability and supports malignant progression (Harris, 2002).

One of the metabolic adaptations enabling survival of cancer cells in a hypoxic environment is the switch from aerobic to anaerobic metabolism. This switch seems to be under the regulation of HIF-1 α (Chiavarina et al., 2012). The expression of genes coding for metabolic enzymes such as GLUT1, ensuring glucose uptake, and lactate dehydrogenase (LDH), favouring anaerobic metabolism, are activated after HIF-1 α stabilisation and nuclear

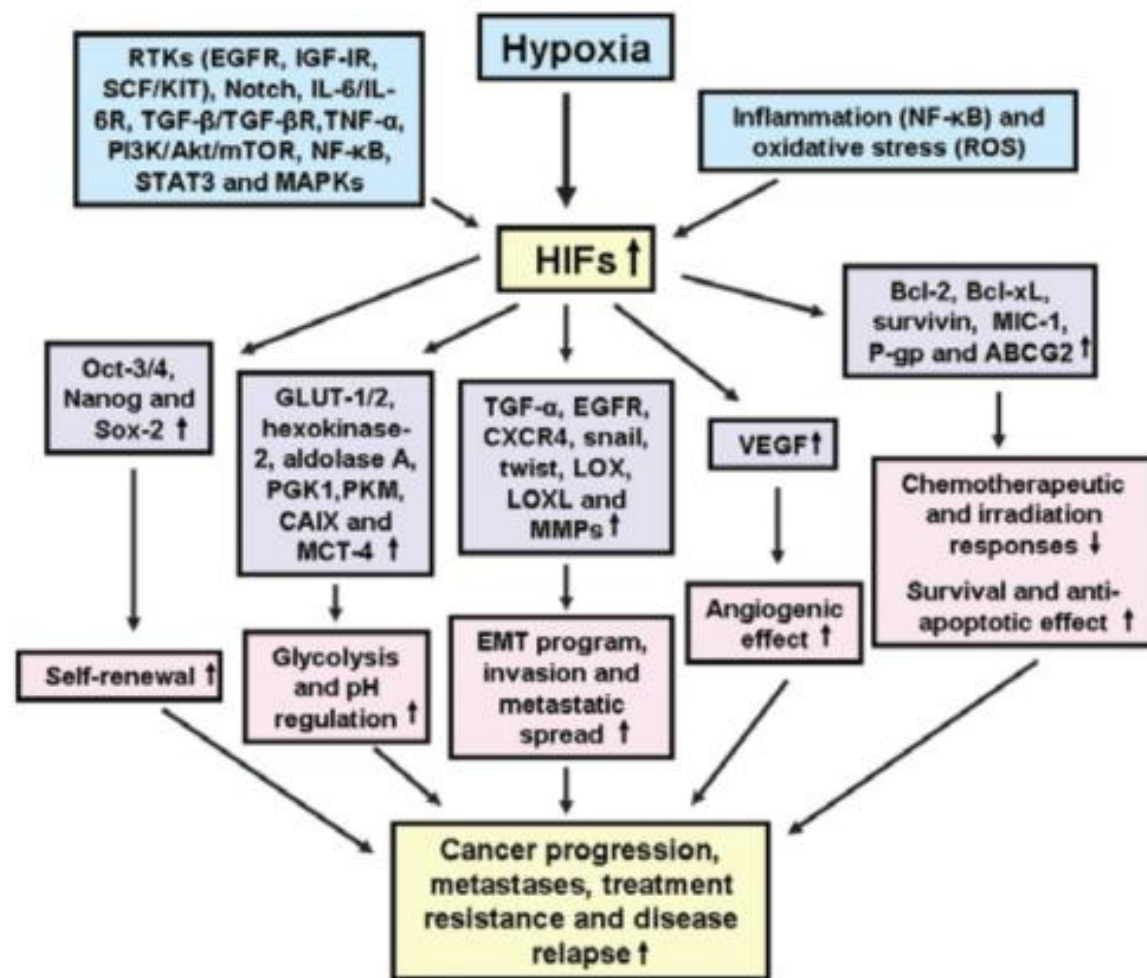


Figure 12. Cellular adaptations leading to cancer progression. HIFs can activate many various pathways that can lead to cancer progression and increased malignancy. This is achieved by the activation of stemness genes, by altering metabolism, inducing EMT, increasing angiogenesis and favouring survival mechanisms. (Mimeault & Batra, 2013)

translocation (Harris, 2002). Via glutaminolysis glutamine is used as a favoured source of energy in cancer cells. This amino acid is used either to regenerate pyruvate via the malic enzyme or, by entering the Krebs cycle, to replenish the intermediates that are necessary to generate lipids, nucleotides and other amino acids required for cancerous cells with high proliferative activity (Hirschhaeuser, Sattler, & Mueller-Klieser, 2011; Upadhyay et al., 2013). Although endowed with a high glucose uptake cancer cells seem to evade the oxygen dependent tricarboxylic acid cycle by favouring the production of lactate from pyruvate via LDH. Directing glucose metabolism toward anaerobic catabolism decreases the overall pH of tumours. For tumours a decreased pH is actually favourable as it stimulates growth and invasion of neighbouring tissues and protects the cancer cells from the immune system. By only undergoing glycolysis and evading the mitochondria completely the cell generates 2 molecules of ATP for each molecule of glucose compared to 36 molecules of ATP produced in the Krebs cycle. This method of metabolism seems to be less efficient but comes with the advantage of generating metabolic intermediates that can be used to synthesize nucleotides, amino acids and lipids. This process occurs in cancer cells as well as in normal proliferating and migrating cells. It can also take place in normoxic cancer cells where it is known as the Warburg effect (Harris, 2002; Upadhyay et al., 2013; Vander Heiden et al., 2009).

The effects of low oxygen can outlast the duration of hypoxia, or even become permanent. This is due to the epigenetic changes caused by hypoxia (Harrison et al., 2012; Upadhyay et al., 2013)

4. The influence of hypoxia on hyaluronan metabolism

Hypoxia can indirectly regulate HA metabolism. For instance, many genes involved in HA metabolism have lactate-sensitive response elements in their promoters (Stern, 2008a). This is the case for CD44, HYAL1, and HYAL2, whose transcriptions are all activated by the by-product of the altered metabolism. Increased expression of CD44 in cancer cells or in the cells composing the tumour microenvironment in turn promotes cancer cell growth and motility (Formby & Stern, 2003; Hirschhaeuser et al., 2011; Stern, 2008a).

Recently it has been discovered that HIF-1 α directly induces the expression of CD44. Not only the standard isoform of CD44 (CD44s) but also two variants, CD44v6 and CD44v8, are up-regulated in response to hypoxia. The alternate splice forms of CD44 are both associated with poor patient outcome, whereas CD44s is considered as a sign of good clinical outcome. CD44's involvement in cellular differentiation creates an indirect tie between hypoxia, HA metabolism and stem cells (Krishnamachary et al., 2012).

5. Hypoxia and cancer stem cells

CSCs seem to have a primordial role in tumour progression (Mimeault & Batra, 2013b). This suggests that they can reside in the hypoxic environments that compose the natural stem cell niche but also arise in solid tumours (Keith et al., 2012; Louie et al., 2010). A study conducted by Mathieu *et al.* shows that there is an expression overlap of the genes involved in stemness when comparing human embryonic stem cells and cancer cells cultured in hypoxia (Mathieu et al., 2012). The hypoxic environment seems to be a crucial factor for all types of stem cells (embryonic, adult, and CSCs) as it maintains pluripotent potential, inhibits differentiation, and maintains the integrity of the genome (Keith & Simon, 2007; Li et al., 2009). HIFs can modify transcription factor expressions and signalling pathways involved in differentiation processes, inducing the maintenance or acquirement of a stem cell phenotype. This is possible by activating the expression of iPSC inducers.

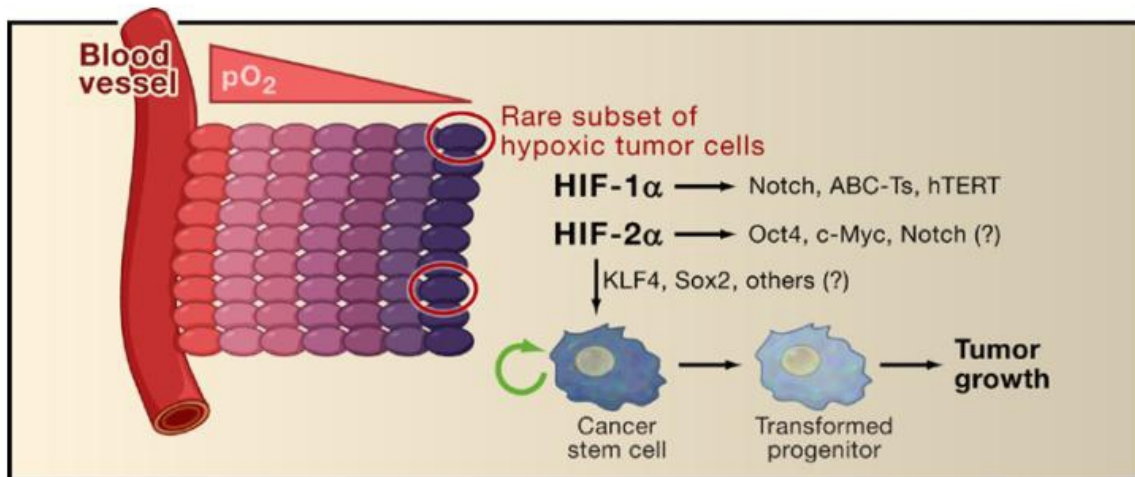


Figure 13. HIF isoform specific stemness associated gene activations. In some hypoxic tumour cells HIF stabilisation and nuclear translocation will induce the transcription of genes involved in the stemness process, such as Notch, ABC-transporter, Oct4, c-Myc and maybe some of the other iPSC inducers too (KLF4, Sox2). The activation of these genes can bestow the cells with stem cell properties, such as self renewal (indicated with a green arrow) or induce dedifferentiation. These cells could have an increased potential to replicate which can lead to the formation of tumours if not brought under control. (Keith & Simon, 2007)

Both HIF-1 α and HIF-2 α are involved in activating genes necessary for stemness such as those coding for Notch or ABC transporters (which bestow the stem cells with their capacity to expulse drugs) but it seems that HIF-2 α may have the predominant influence on limiting differentiation. HIF-2 α directly activates two of the four main stemness transcription factors, Oct4 and c-Myc, but not the remaining two factors, KLF4 and Sox2. To this day HIF-2 α is still poorly understood and could possibly offer more insight into cancer initiation through CSCs (Fig. 13) (Keith & Simon, 2007). HIF-1 α is thought to be primarily involved in inducing neo-angiogenesis and directing the CSCs to express the enzymes necessary for anaerobic metabolism (Keith et al., 2012).

ALDH, a CSC biomarker, is highly up-regulated in many BCs and can be linked to poor clinical outcome. Genomic analyses indicate that this enzyme can be highly expressed during oxidative stress, which can occur after re-oxygenation of a hypoxic environment. Proliferation and differentiation of mesenchymal stem cells has been associated to a high ALDH activity in a hypoxic environment. This indicates that there is a correlation between hypoxia, ALDH and CSC. Further studies in this domain showed that ALDH activity can indeed activate HIF-2 α (Kim et al., 2012).

Hypoxia is thought to induce CSCs to undergo epithelial-mesenchymal transitions (EMT), a process that is critical for metastatic activity. Cancer cells that have undergone EMT can be characterised as having increased migratory and invasive abilities. In invasive breast tumours the molecular process leading to this change in phenotype is thought to be caused by a hypoxia induced up-regulation of Jagged2, which in turn activates the Notch pathway involved in stemness and EMT (Mimeault & Batra, 2013a, 2013b). CSCs with an EMT phenotype in hypoxic conditions are able to maintain this status even after re-oxygenation, thus maintaining their ability to migrate. Differentiated cells are also able to undergo EMT but after re-oxygenation these cells revert to their epithelial status via mesenchymal-epithelial transition. Cells cultured in non-adherent conditions can be enriched in CD44⁺/CD24⁻ cells after multiple re-oxygenation cycles. Within these cell cultures hypoxia can activate the Wnt/ β -catenin pathway as well as the PI3K/Akt cascades. These studies have revealed that this activation confers CSCs with higher MS-forming and clonogenic abilities (Mimeault & Batra, 2013a, 2013b).

The hypoxic environment can drive cancer progression by interfering with cellular communication. The hypoxic zones observed in breast tumours but also naturally found in the bone can promote bone metastasis by modifying cytokines (SDF-1) and growth factors (Bone Morphogenic Protein (BMP) and TGF- β 1) released by stromal cells and BC cells. These molecules can influence self-renewal, survival, dormancy, and the ability of breast CSC to generate osteolytic bone metastasis (Mimeault & Batra, 2013a, 2013b).

Not all CSCs will respond to hypoxia in the same way; that will depend on certain protein expressions such as those of ER- α and E-cadherin (Chu et al., 2013; Harrison et al., 2012). E-cadherin is a protein involved in cell-cell interactions, and its expression is lost during EMT. This change of phenotype enables migratory events necessary for metastasis in hypoxic conditions, but only in CSCs presenting low E-cadherin and high vimentin expressions (Salnikov et al., 2012). CSCs issued from ER- α positive cell lines have higher MS-forming abilities than those from ER- α negative cell lines (Harrison et al., 2012). It is not yet entirely determined what mechanisms are involved in increasing the fraction of undifferentiated cells. There are indications that self-renewal pathways are altered and that a certain proportion of dedifferentiation occurs in ER- α positive cells, leading to an increased proportion of CSCs. In the ER- α negative cell lines it is thought that hypoxia modifies proliferation and differentiation, causing a reduced number of CSCs (Harrison et al., 2012). This is merely a slight indication of how complex the effects of hypoxia on cancer progression are. A more in-depth comprehension of the underlying molecular mechanisms is

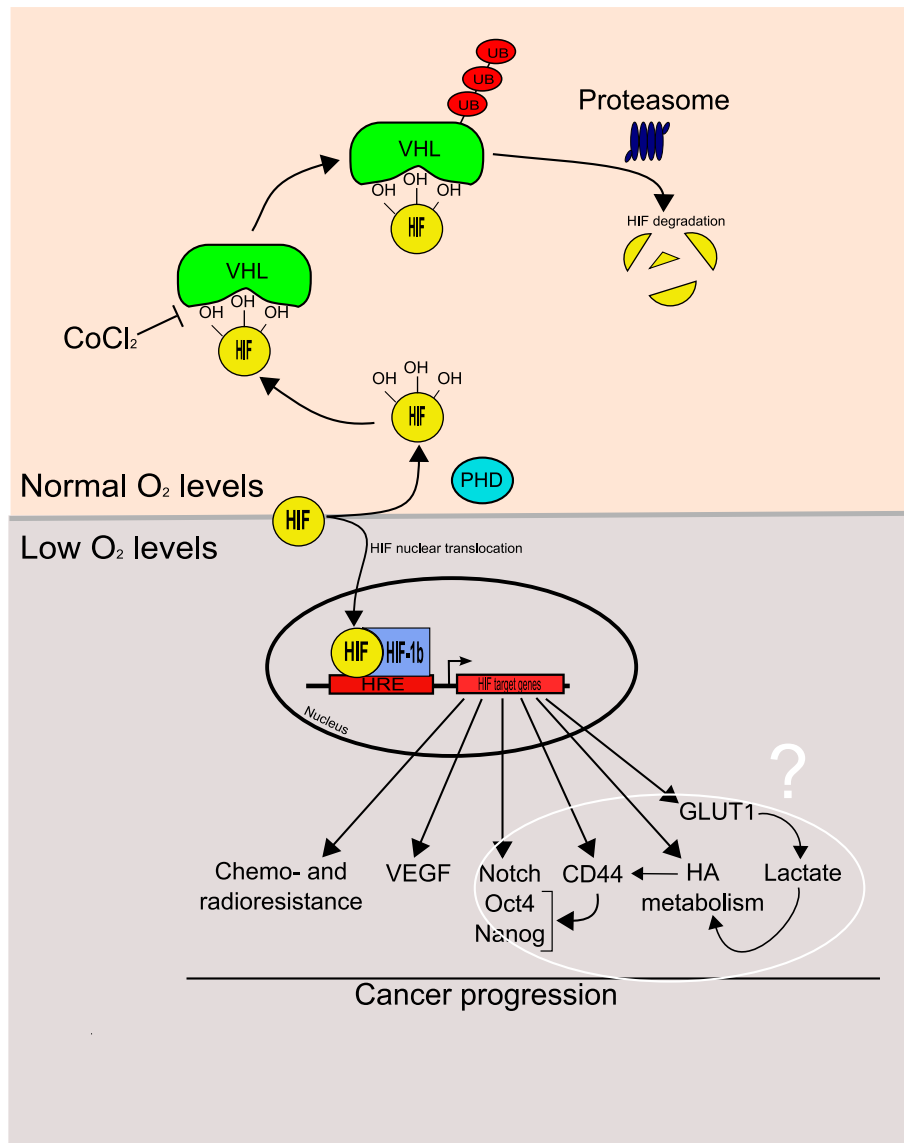


Figure 14. Contribution of hypoxia-inducible factors (HIF) to cancer progression. In oxidative conditions, as is the case in normoxia, the HIF- α subunits undergo hydroxylation via prolyl-hydroxylase (PHD). In this hydroxylated state the α -subunit can bind to Von Hippel Lindau E3 ubiquitin ligase (pVHL) which is then ubiquitinated. The complex is thus targeted to the proteasome for degradation. During hypoxia, a reducing environment, the HIF- α subunits undergo redox-dependent stabilisation and independently translocate to the nucleus, thus avoiding proteolytic degradation. After this nuclear translocation HIF- α can interact with oxygen independent HIF-1 β and activate gene transcription. These two subunits form a complex that can bind to hypoxia-response elements (HRE). HIF-1 α , induced by severe oxygen deprivation, and HIF-2 α , induced by moderate hypoxia, are highly homologous proteins. They share many common HREs but some are isoform specific. Once the complex is bound to the HRE gene transcription is initiated. Some of the genes activated by HIFs are VEGF, Notch, Oct4, Nanog, HA metabolisers, CD44 and GLUT1. Genes promoting chemo- and radioresistance are also activated. The activation of these genes contributes to cancer progression. The area encircled in white is still currently being researched; the individual elements have been examined but not altogether in the same context. Adapted from (Chilov et al., 1999; Krishnamachary et al., 2012; Li et al., 2009; Mimeault & Batra, 2013).

crucial for the development of effective cancer treatments but is beyond the scope of this introduction.

6. Hypoxia, hyaluronan metabolism and cancer stem cells

Very few studies have tried to elucidate the link between HA metabolism, hypoxia and CSCs (Krishnamachary et al., 2012). Most studies focused on two of these elements and limited themselves to briefly mentioning the remaining factor (Darzynkiewicz & Balazs, 2012; Williams et al., 2013). Nonetheless there is ample evidence of a tie between both microenvironmental factors (HA and hypoxia) and CSCs. Briefly, HA metabolism as well as its ability to create an embryonic-like environment are highly involved in regulating the differentiation processes (Bourguignon et al., 2008; Charafe-Jauffret et al., 2008; Darzynkiewicz & Balazs, 2012; Keith & Simon, 2007; Stern, 2005). The lactate produced under hypoxia can stimulate HA metabolism (Stern, 2008a). HIF-1 α can also directly affect HA metabolism by inducing the expression of CD44 or changing the proportions of standard and variable CD44 isoforms (Krishnamachary et al., 2012). The fact that CSCs usually present a CD44⁺ phenotype is further proof that HA metabolism may play an important role in CSC biology (Stern, 2005). Both hypoxia and HA metabolism seem to be tied to the acquirement of a stem cell phenotype and the persistence of CSCs, be it by altering normal stem cells or by inducing the acquirement of a stem cell phenotype in already differentiated cells. Any alteration to the pathways deemed to control the balance between symmetric and asymmetric divisions, and to regulate proliferation, dormancy and cell survival, can lead to tumour initiation, maintenance and progression (Fig. 14).

All these elements lead to the construction of the objectives of the current research project.

III. Objectives

The study carried out throughout this Master's Thesis aims to investigate the link between hypoxia, the metabolism of HA and CSCs. CSCs are implicated in both the initiation of tumour development and its growth and progression towards a malignant phenotype. Hypoxia and HA metabolism can individually impact cancer progression but it is becoming clear that they can act together to alter the cellular pathways of CSCs supposedly bestowing them with abilities associated with a more aggressive phenotype.

We decided to isolate and study the CSCs from two different breast BC epithelial cell lines, MCF-7 and MDA-MB-231. The former are of luminal origin and express ER- α . This cell line is not considered as possessing a particularly malignant phenotype. The MDA-MB-231 cells are of basal origin. They do not express ER- α , progesterone receptor, or Her2 antigen, which leads them to be known as triple negative cells. This triple negative status bestows them with a particularly malignant phenotype. Both cell lines form monolayers, with a population of cells composed of differentiated as well as undifferentiated cells. The rare CSC and early progenitor cells are comprised in the undifferentiated cell component.

Many models exist to isolate CSCs but the one chosen for the current studies is the MS forming assay. These roughly spheric formations are initiated by a single isolated CSC or early progenitor cell, then grow and enrich the CSCs, and finally, over time, differentiate to form heterogeneous structures representing solid *in vivo* breast tumours. Of course this model does not replace the physiological environment, but it allows researchers to test the effect of individual and combined factors on the biology of the cells composing these three-dimensional structures. This method of CSC enrichment via MS formation is representative of *in vivo* tumours, is ethically favourable, more cost effective and simpler to carry out than the

transplantation of CSCs into immunodeficient mice. The MS model makes it possible to explore the effects of one external factor, notably hypoxia, on MS formation and on HA metabolism, by investigating the changes in expression of the enzymes responsible for HA synthesis, HAS2 and HAS3, those responsible for HA degradation, HYAL-1 and HYAL-2, and the main receptor for HA and a stem cell marker, CD44, that hypoxia could induce in the chosen CSC model. HAS1 was not studied as previous results conducted in the laboratory indicated that this enzyme is not detectable in MDA-MB-231 and MCF-7 cell-lines.

There was a certain degree of fine tuning that had to be executed, especially regarding the cellular density for optimal MS seeding. The results that will be presented are issued from these optimised seeding densities and conditions. I will not detail the preliminary experiments. The MSs are cultured for 4 and 7 days in normoxic or hypoxic conditions. Thereafter the effects of different oxygen concentrations on MS formation and on the mediators of HA metabolism are evaluated with multiple techniques such as MS counts, immunofluorescent staining, RT-qPCR analyses, western blot analyses and HA dosage. As the aim of the study is to check how CSCs and their HA metabolism can be altered depending on their oxygen environment, the results obtained with hypoxic MSs are compared to the normoxic MSs at the two mentioned stages of development.

This master's Thesis should elaborate how the metabolism of HA in CSCs is modified by hypoxia. This may provide us with insight on how these two factors, HA and hypoxia, may drive cancer initiation and progression.

IV. Materials and Methods

A. Adherent cell lines

Two cell lines, MCF-7 and MDA-MB-231, are used for the studies. The parental cell lines MCF-7 and MDA-MB-231 are initially grown in adherent conditions in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) containing 10 % inactivated FBS (Foetal Bovine Serum) (Lonza) and 15 mM of Hepes buffer (Lonza). FBS inactivation is accomplished by incubating the serum for 30 min at 50 °C. The cells are passaged twice a week and passaging is limited to approximately 25 times. First the adherent cells are rinsed with 4 ml of Phosphate Buffer Saline (PBS). PBS is composed of KH_2PO_4 1.54 mM, Na_2HPO_4 2.7 mM and NaCl 154 mM diluted in water and is adjusted to a pH of 7.2. 1 ml of Trypsin-EDTA (Lonza) is added to the monolayer and incubated at 37 °C for 5 min. The cells are resuspended in 4 ml of the complete DMEM medium, which also inactivates the Trypsin and then centrifuged at 200 xg for the duration of 5 min, after which the supernatant containing the Trypsin is removed. The cells are resuspended in 6 ml of complete DMEM medium and seeded at the wanted density. The newly passaged cells are incubated at 37 °C with 5 % CO_2 .

B. Mammosphere culture

The generation of MSs is achieved by seeding the parental cell lines, pre-incubated in normoxia or for 48 h in severe hypoxia, in non-adherent conditions. This is done when the monolayer of adherent cells has reached 80 % confluence. The non-adherent environment is provided by using Ultra low adherence 6-well plates (Corning) or Petri dishes coated with PBS-Agarose 1%. The base medium in which the cells are seeded is composed of MammoCult medium basal (Stemcell Technologies) which is supplemented with 10 % MammoCult proliferation supplements, (Stemcell Technologies) heparin 4 µg/ml, hydrocortisone 0.48 µg/ml (StemCell), and L-glutamine 5 µl/ml (Glutamax 100X Gibco), is added. First the cells must be detached by adding 1 ml of Trypsin to MCF-7 and 1 ml of Accutase (Sigma) to MDA-MB-231. After 5 min of incubation at 37 °C both cell lines are suspended in 2 ml of MammoCult medium. The cell suspension is centrifuged at 200 xg for 5 min after which the supernatant containing the trypsin or accutase is removed. It is vital that the cells are adequately dissociated, which is achieved by repeated pipetting; 40 times for the MCF-7 cell line and 20 times for the MDA-MB-231 cell line. By using the TC10TM automated cell counter (Bio-Rad), which counts live and dead cells, it is possible to determine the volume of isolated cells needed to plate the MSs at the correct concentration. The isolated cells are diluted in the complete medium MammoCult and seeded at the correct cellular density.

Two different cell dilutions, 1 000 and 5 000 cells per ml of MammoCult medium, are used to generate the MSs. Ultra low adherence 6-well plates (Corning) are used for the 1 000 cells per ml seeding, of which the generated MSs are used for MS counts. PBS-Agarose 1 % coated Petri dishes are used to grow the MSs at a density of 5 000 cells per ml. These MS are used for immunofluorescent staining, RNA and protein assays, pH measurement of the medium and hyaluronic acid dosage of the medium and of the cells composing the MS. The MS are cultured for a total of 4 or 7 days after the initial seeding in either normoxic or hypoxic conditions.

Staining	Permeabilisation	Aspecific binding blocking		Primary AB			Secondary AB			
		BB	Time	AB	AB dilution	Time	AB	Dilution	Fluorochrome	Time
HYAL-2	0,5 % Triton X-100 -PBS (5 min)	10 % goat serum- PBS – BSA 0.1%	30 min	Rabbit anti-HYAL-2	0,016 µg/µl - BB	OV at 4 °C	Goat anti-rabbit IG	1/500 - BB	Alexa Fluor-488	1h RT

Table 3. Summary of incubation durations and antibody concentrations for the immunofluorescent staining. AB = Antibody, BB = Blocking buffer, OV = Overnights, RT = Room temperature, IG = Immunoglobulins

C. Normoxic and hypoxic cultures

The adherent cell culture of both MDA-MB-231 and MCF-7 cells are cultured in normoxic conditions, or are incubated in severe hypoxic conditions (1 %) for 48h prior to MS seeding. After seeding, MSs are cultured either in normoxic conditions, 20 % O₂ and 5 % CO₂ at 37 °C or in severe hypoxic conditions 1 % O₂ and 5 % CO₂ at 37 °C. The hypoxic environment is created by pumping nitrogen into a hypoxia chamber (BioSpherix) placed within an incubator at 20 % O₂ and 5 % CO₂ at 37 °C.

D. Mammosphere count

The MDA-MB-231 and MCF-7 MSs seeded at a density of 1 000 cells per ml in Costar 6 well plates are used for the MS count. In order to carry out a count of the number of MSs contained in one well, the MSs are transferred with much precaution, 500 µl at a time, into a well of a 24-well. In order to limit the dissociation of the MSs whilst transferring them from one well to another, approximately 5 mm of the end of the tips of the micropipette are cut off before hand. MS collected after rinsing the well with 500 µl of PBS are also counted. In order to be counted as a MS, the formation must be composed of at least 5 cells. The adherent culture of MDA-MB-231 is grown in normoxic conditions, and the MS culture is grown in normoxia or severe hypoxia. Before seeding the MSs in normoxia or severe hypoxia the MCF-7 parental cell line is cultured either in normoxia or for 48 h prior to seeding in severe hypoxic conditions.

E. Immunofluorescence

For the immunofluorescent study MSs of MDA-MB-231 and MCF-7 cells are seeded at a density of 5000 cells per ml in a 20 cm² PBS-Agarose 1% coated Petri dish (one dish per condition). The MSs are first centrifuged at 15 xg for the duration of 5 min. After removal of the supernatant the cells are resuspended in 100 µl of PBS. The MSs are gently dissociated by pipetting, taking care not to damage the cells. As MSs do not adhere to glass slides they must first be projected onto glass slides using a CytospinTM system after which they are fixed with paraformaldehyde 4 % for 10 min. The slides are then rinsed with PBS two to three times after which the staining procedure can begin.

An immunofluorescent staining of HYAL2 is produced in parallel to a nucleus staining with TOPRO-3. The cells now fixed to the slides are first permeabilised and then aspecific antibody binding is blocked for 30 min in the blocking buffer. After three 5 min rinses with PBS the specific primary antibody can be applied. After PBS rinsing the secondary fluorochrome-coupled antibody directed against a species specific immunoglobulin is added to the slides. TOPRO-3 diluted 1/80 in PBS-RNase 2 mg / ml is applied after slide rinsing. The slides are then mounted in Mowiol. Table 3 summarizes the different solutions, antibodies, dilutions and incubation durations necessary for the immunofluorescent staining.

F. Protein extraction and western blotting

MDA-MB-231 and MCF-7 MSs cultured at a density of 5000 cells per ml in normoxia and severe hypoxia for 4 and 7 days are collected from 3 Petri dishes. Each dish is rinsed out with 2 ml of PBS to insure that all MSs have been acquired. The MSs are first centrifuged at 15 xg for 5 min after which the supernatant is removed. The cells are resuspended in 200 µl of PBS and transferred to an eppendorf which is centrifuged at 1 800 xg for 5 minutes. The supernatant is entirely discarded, and the dry cell pellet is stored at -80 °C.

1. Protein extraction and dosage

The cell samples are lysed using 500 µl DIGE labelling buffer also known as DLA (Thiourea 2 M, Urea 7 M, CHAPS 4 %, Tris 30 mM, pH 8.5). This buffer also contains a protease inhibitor cocktail (Roche) at a concentration of 40 µl/ml. After this the sample is homogenised and left to incubate for 20 min on a wheel at room temperature. Next the samples are sonicated at a low amplitude 3 times for 10 sec. After sonication the samples are centrifuged for 10 min at 12 500 xg. The supernatant is delicately collected, paying attention not to touch the viscous pellet. A fraction of the supernatant is dosed using the Pierce 660 nm protein assay reagent (Thermo Scientific) in order to determine the concentration of proteins in each sample.

2. Sample preparation

15 µg of proteins are used when assaying HYAL1 and 10 µg are used for HYAL2. To do so the appropriate volume of the extracted protein sample is diluted in water. 5 µl of blue 5X (Bromophenol blue, glycerol, SDS and TRIS-HCl) containing 1/20 β-mercaptoethanol is added, in order to weigh down the proteins and provide them with a negative charge that is proportional to their molecular weight. The final volume must be 40 µl. By boiling the samples for 3 min at 100 °C the proteins are denatured. The samples are now ready to be loaded into a track of a 1.5 mm Acrylamide Bisacrylamide (Bio-Rad) 10 % gel.

3. Polyacrylamide gel preparation

The polyacrylamide gel used for these protein assays has a thickness of 1.5mm and contains 10 % Acrylamide bisacrylamide. First the running gel has to be cast. This gel is composed of 2.5 ml of TRIS-HCl 1.5M pH 8.8 SDS 0.4 %, 2.5 ml of Acrylamide Bisacrylamide 40 %, 4.5 ml distilled water, 500 µl Ammonium persulfate (Bio-Rad) and 10 µl TEMED (Bio-Rad). As oxygen inhibits polymerisation a layer of ethanol is poured over the freshly cast gel. The gel polymerises after 30 min, it is at this point that the ethanol will be removed and the stacking gel can be cast. The stacking gel is composed of 1.25 ml of TRIS-HCl 0.5M pH 6.8 SDS 0.4 %, 475 µl of Acrylamide Bisacrylamide 40 %, 2.9 ml distilled water, 375 µl Ammonium persulfate and 5 µl TEMED. Spacers are placed into the stacking gel in order to create tracks in which the prepared protein samples can be loaded. The stacking gel also takes approximately 30 min to completely polymerise. The gels can be used straight away or stored at 4 °C for later use.

4. Protein loading and electrophoresis

The gels are placed in the electrophoresis buffer tank which is filled with the migration buffer (TRIS-HCl 25 mM, pH8.3, Glycine 192 mM and SDS 0.1 %). In one of the tracks 15 µl of Protein Markers (ProSieve) is added which will form a molecular weight ladder after the electrophoretic migration and in the others 40 µl of the prepared protein samples are loaded. Migration and separation of proteins are induced by electrophoresis. Initially the migration is performed at 80 V for a duration of approximately 20 min, which ensures adequate stacking of the proteins. Afterwards the voltage is increased to 120 V. The migration is performed during approximately 1 h 30 min in the migration buffer.

	PM (kDa)	Primary AB		Secondary AB		Blocking Solution	Solution for primary - and secondary AB dilutions
		Dilution	Incubation	Dilution	Incubation		
HYAL1	48	1 / 250	Overnight 4 °C	1 / 4 000	1 h	Blotting solution 5 % - TBS- Tween 1 %	TBS- Tween 1 %
HYAL2	54	1 / 1 000	Overnight 4 °C	1 / 4 000	1 h	Blotting solution 5 % - TBS (Abcam) - Tween 1 %	TBS (Abcam)- Tween 1 %
Actin	43	1 / 100 000	30 min	1 / 4 000	45 min	Blotting solution 5 % - PBS- Tween 1 %	PBS- Tween 1 %

Table 4. Summary of incubation durations, antibody dilutions and blocking solutions for the Western blot. Blotting solution is supplied by Roche.

5. Semi-humid transfer

After the molecular weight-specific separation induced by electrophoresis, the proteins that are contained in the gel are transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-humid method. The PVDF membrane is soaked for 15 sec in methanol 100 % and then conditioned for 10 min in a transfer buffer (TRIS-HCl 2.5mM pH 8.3, Glycine 192mM, methanol 20 %). For each gel 6 pieces of Whatman paper is needed. These sheets are soaked in the transfer buffer. 3 of the sheets are stacked on the tray of the machine that will supply the electric current necessary for the transfer. Consecutively the PVDF membrane is placed on the Whatman paper, the gel with the migrated proteins and finally the 3 remaining sheets are stacked on top. The transfer is performed in the 20 % methanol Tris-Glycine buffer and is performed for the duration of 1 h 30 min. The transfer machine is set to 60 mA per PVDF membrane and 20 V which is independent of the number of PVDF membranes used.

6. Protein detection

After the transfer the protein of interest and a protein for normalisation, in this case actin, can be detected by the use of antibodies combined with chemiluminescence. First nonspecific antibody binding is blocked by incubating the PVDF membrane with Roche's chemiluminescence supplied blocking reagent diluted to 1 % in Tris Buffer saline (TBS) (Abcam) containing 0.1 % Tween. The membrane is then rinsed repeatedly for 30 min. A rabbit produced primary polyclonal antibody against HYAL1 (Sigma) or HYAL2 (Abcam) is added and left to incubate at 4 °C overnight. The membrane must thereafter be washed repeatedly for 30 min with the adequate buffer (PBS- or TBS- Tween 1 %). The membrane is then incubated with a goat produced secondary antibody directed against rabbit immunoglobulins. This antibody is coupled to horseradish peroxidase (HRP) which catalyzes a light emitting reaction once the luminescence substrate has been added. This emission of light is detectable by photographic films or phosphor-imaging screens. Both of these antibodies are diluted in 0.5 % blocking reagent – TBS – 0.1 % Tween. The proteins are now detectable via the emission of luminescence. In the dark room a film is placed on top the membrane to which the luminescence substrate has been added. The film is exposed for a certain duration after which the film is developed. Bands proportional to the quantity of the protein of interest should appear on the film after development. Next the membrane is incubated with the primary antibody directed against actin that is produced in the mouse. Thus the secondary antibody is an anti-mouse immunoglobulin antibody. Both of these antibodies are diluted in the 1 % blocking reagent – PBS – 0.1 % Tween. By adding the HRP substrate light will be emitted by any secondary antibodies that have bound to the membrane. Again the quantity of protein can be revealed by exposing photographic film to the light emitting membranes. After this a semi-quantative study of the expression of the protein of interest is enabled. The protein of interest is normalised to the abundance of actin. Table 4 shows a summary of the antibodies and incubation durations used.

G. RNA extraction and Real-time reverse-transcription PCR

MDA-MB-231 and MCF-7 MSs grown in normoxia and severe hypoxia are recovered from 3 Petri dishes at 4 and 7 days, and are each rinsed out with 2 ml of PBS. After a 5 min round of centrifugation at 15 xg, the supernatant is removed and the MSs are resuspended in 500 µl of TRI Reagent (Sigma) and stored at – 80 °C until needed for RNA extraction.

	Forward primer (5' - 3')	Reverse primer (5' - 3')
HAS2	CCTCATCATCCAAAGCCTGT	GATGCAAAGGGCAACTGTTT
HAS3	GCCCTCGGCGATTCTG	TGGATCCAGCACAGTGTCTAGA
CD44	CATTGCAGTCAACAGTCGAAGAA	ATTGCCACTGTTGATCACTAGCTT
Actin	CTGCTACCAGGACACGATTT	CCCTTACACATCGGAGAACAG

Table 5. Primer sequences for RT-qPCR

1. RNA extraction

The RNA extraction is conducted in RNase free conditions. After homogenisation the samples are incubated for 5 min at room temperature, then 100 µl of chloroform is added and the tubes are briefly vortexed. The specimens are centrifuged at 12 000 xg for 15 min at 4 °C. The aqueous phase is recovered and the rest is discarded. The next steps of the RNA extraction follow the protocol of the RNeasy minElute Cleanupkit (QIAGEN). First 300 µl of ethanol 70 % is applied and mixed by pipetting then the sample is transferred to a 2 ml RNeasy MinElute spin column. The tubes are centrifuged at 7 400 xg for 15 sec after which the flow-through is discarded. Next 500 µl of the supplied RPE buffer is added and centrifuged for 15 sec at 7 400 xg. 500 µl of ethanol 80 % is applied to the spin column and is then centrifuged for 2 min at 7 400 xg. The spin column is then placed in a new collection tube, with the lid open, and is centrifuged for 5 min at full speed. After discarding the flow-through the column is placed in a new 1.5 ml collection tube. 20 µl of RNase-free water is applied directly to the centre of the spin column membrane and is then centrifuged at full speed for 1 min in order to elute the RNA. The flow-through is reapplied to the spin column membrane and centrifuged another time, which insures that all the RNA has properly detached from the membrane and eluted into the collection tube. The RNA obtained from this extraction can be quantified using the Nanodrop, which determines the concentration on the basis of spectra absorption at 260 nm.

2. Retro-transcription and quantitative PCR (RT-qPCR)

The qRT-PCR is accomplished in two steps. The extracted RNA, which can be stored at -80 °C until needed, is retro-transcribed into cDNA. To this purpose a volume containing 0.5 µg of RNA is added to an eppendorf to which a certain volume of RNase free water is added in order to come to a final volume of 13 µl. The samples are heated for 10 min at 70 °C and thereafter put on ice. The retro-transcription is carried out by adding 4 µl of M MLV-RTase Buffer (Promega), 1 µl dNTP 10 mM (Promega), 1 µl of Random hexamers (Invitrogen) diluted 10x and 1 µl of M MLV-RTase (Promega) (kept on ice) to each sample. The specimens are then placed into a thermal cycler that effectuates a single cycle of 5 min at 25 °C, 60 min at 42 °C and finally 5 min at 95 °C. The samples are stored at 4 °C or directly used for the PCR.

For the second step of the PCR, the obtained cDNA is diluted 10x in RNase free water. 5 µl of the cDNA is then deposited into a well of a 96-well PCR plate. To each well containing cDNA 10 µl of Sybrgreen, 2.5 µl of the forward primer and 2.5 µl of the reverse primer of the gene of interest are added. The primers have a final concentration of 0.3 µM. Every condition is done in triplicates. The plate is centrifuged at 295 xg for 3 min after having been sealed with a thermal seal. The plate is then introduced into the thermal cycler for 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. For the primer sequences see Table 5.

H. PH measurement

The medium of MDA-MB-231 and MCF-7 MSs cultured for 4 and 7 days in normoxic or severe hypoxic conditions is collected after centrifugation at 15 xg for 5 min. The pH of the medium is then directly measured with a pH meter.

I. Hyaluronic acid dosage

1. Sample preparation

The dosage of HA is carried out on MS obtained after having been cultured for 4 and 7 days in either normoxia or hypoxia. A total of 4 PBS-Agarose 1% coated Petri dishes containing MDA-MB-231 MSs seeded at a density of 5 000 cells/ml are needed for each condition.

The day prior to MS collection (third day for MSs collected at developmental day 4, and the sixth day for MSs collected at developmental day 7) the medium containing the spheres of each individual condition is collected and pooled. The Petri dishes are each rinsed with 5 ml of PBS after which the solution is also collected, in order to recover any remaining MSs. The samples are then centrifuged at 15 xg for the duration of 5 min. The supernatant is discarded and the MSs are gently resuspended in a total volume of 10 ml of complete MammoCult medium. The resuspended MSs are then reseeded in 1 Petri dish per condition. This precaution is performed in order to be able to study the 24 hour production of HA by the MSs at developmental day 4 and 7 in normoxia or hypoxia. At seeding day 4 and 7 the medium containing the pooled MSs is collected. The Petri dishes are rinsed with 2 ml of PBS and collected. The samples are centrifuged at 200 xg for 5 min after which the supernatant is kept and stored at -20 °C. The pellet of MSs is resuspended in 200 µl of PBS. 170 µl of the sample is transferred to an eppendorf with a screw-top which will be used for the HA dosage within the cell fraction, and the remaining 30 µl is transferred to an eppendorf for protein dosage. The protein dosage is used later on for the normalisation of the results. Both sets of eppendorfs are centrifuged at 1 800 xg for 5 min. The supernatant is then removed from each sample. The fractions destined for the protein dosage are stored at -80 °C. The cell fractions which will be used for the HA dosage are resuspended in 100 µl of TRIS HCL 0.1 M CaCl₂ 10 mM and 11 µl of Pronase (Sigma). The addition of pronase is necessary for the cellular fraction as it will degrade any proteins that may be holding HA, thus allowing its liberation. Next they are incubated for 24 h at 55 °C on a wheel. After the 24 h incubation period the samples are boiled for 10 min and then stored at -20 °C until needed for the HA dosage.

2. Dosage of hyaluronic acid

By using the Hyaluronan Duo Set Kit (R & D Systems) the quantity of HA in the cellular fractions and that that is released into the medium can be dosed. The evening before the HA dosage, 96-well microplates must be coated with 50 µl of a Capture Reagent, made up of Aggrecans diluted in PBS. The plates are sealed and left to incubate at room temperature overnight. The wells are rinsed out three times with a Wash Buffer (PBS 0.05 % - Tween 20), during this process it's important that all the liquid be removed. Next the wells are blocked with the Blocking Buffer (PBS 0.5 % - Tween 20 NaN₃) for 1 h at room temperature. The plates are now rinsed three times after which the plates are ready to receive the sample. The samples and the standards are diluted in the Reagent Diluent (PBS 5 % - Tween 20) after which 50 µl are deposited into each well. The plates are covered and left to incubate for 2 h at room temperature. The plates are rinsed three times, and then 100 µl of the Detection Reagent, composed of biotinylated aggrecans, is added to each well which is then incubated for the duration of 2 h at room temperature. The plates are washed after which 50 µl of Streptavidin-HRP is deposited into each well. The plates are covered and incubated for 20 min at room temperature. The plates should be kept out of direct light. After rinsing the wells three times, 50 µl of the Substrate Solution is added and left to incubate for 20 min. By adding the Substrate Solution the colorimetric reaction induced by the HRP activity is initiated. The

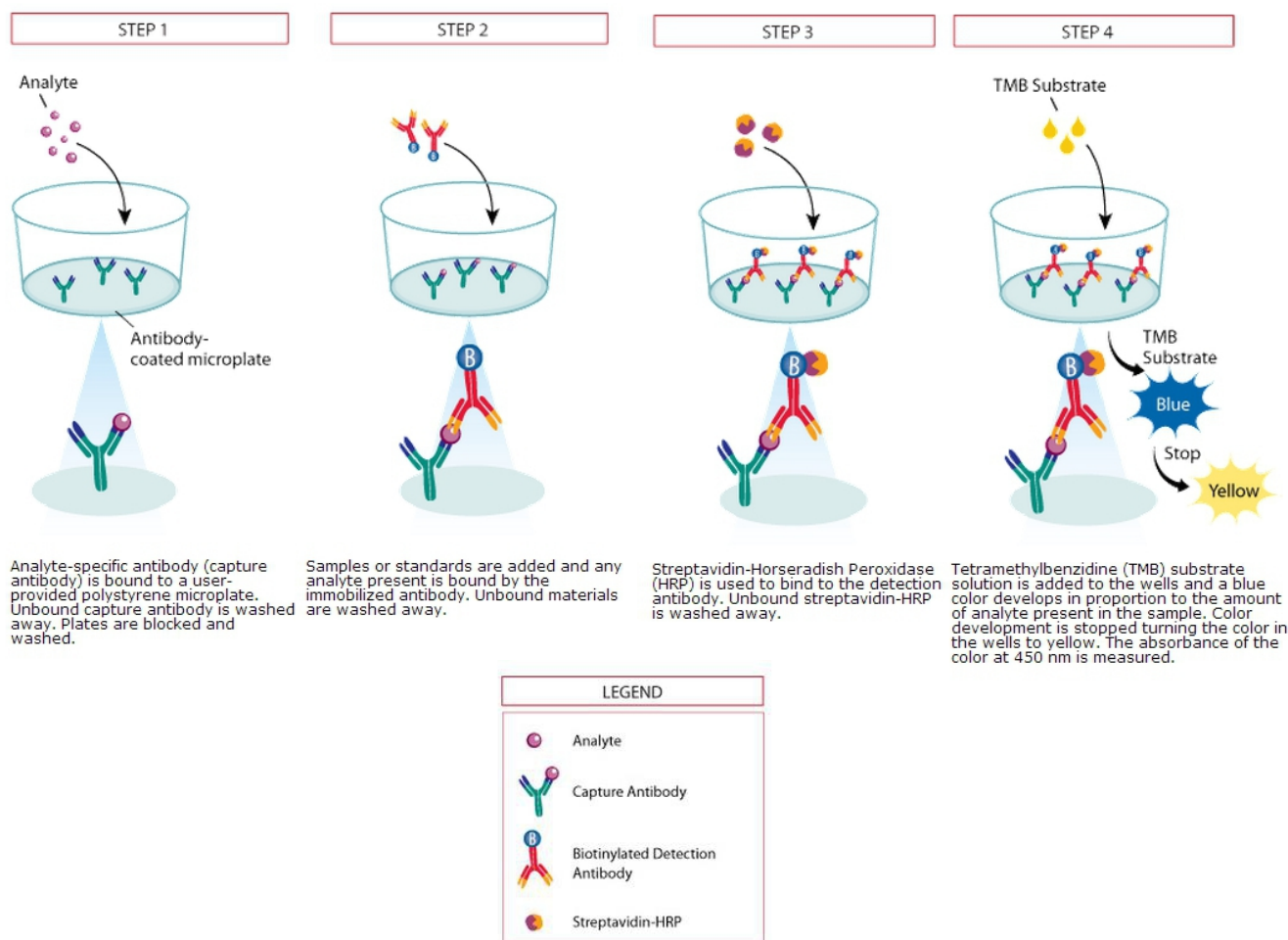


Figure 15. DuoSet ELISA Development Systems Assay Principle. This figure represents the procedure for the HA dosage. In a first step the analyte, in this case HA, will bind to the aggrecans present on the plate. In step 2 the biotinylated aggrecans are added which will bind to the HA already bound to the plate. Step 3, streptavidin coupled to HRP is added which will bind to the aggrecans. In step 4 the HRP substrate is added, inducing a colorimetric reaction. By binding to the biotinylated aggrecan the quantity of HA can be measured, as the HRP induced colour change is proportional to the amount of HA present in the well. The Stop solution blocks the colorimetric reaction and provokes a colour change, turning it to yellow which is detectable at 450 nm. The obtained values are normalised to the protein fractions collected in parallel to the cellular fractions and medium. (Images acquired from <http://www.rndsystems.com>)

plates must yet again be sheltered from direct light. 25 µl of Stop Solution, composed of H₂SO₄, is added to each well, which is well mixed by gently tapping on the plates. This solution stops the colorimetric reaction, thus allowing a spectrophotometric measurement of the optical density for each individual well by using a microplate reader at 450 nm. This procedure enables an evaluation of the quantity of HA present in the cellular fractions and the medium in which the MSs were cultured.

In a first step the HA will bind to the aggrecans present on the plate. After the rinses biotinylated aggrecans are added which will bind to the HA already bound to the plate. Next streptavidin coupled to HRP is added which will bind to the aggrecans. The colorimetric reaction is induced by addition of the HRP substrate. By binding to the biotinylated aggrecan the quantity of HA can be measured, as the HRP induced colour change is proportional to the amount of HA present in the well. The Stop solution blocks the colorimetric reaction and provokes a colour change, turning it to yellow which is detectable at 450 nm. The obtained values are normalised to the protein fractions collected in parallel to the cellular fractions and medium (Fig. 15).

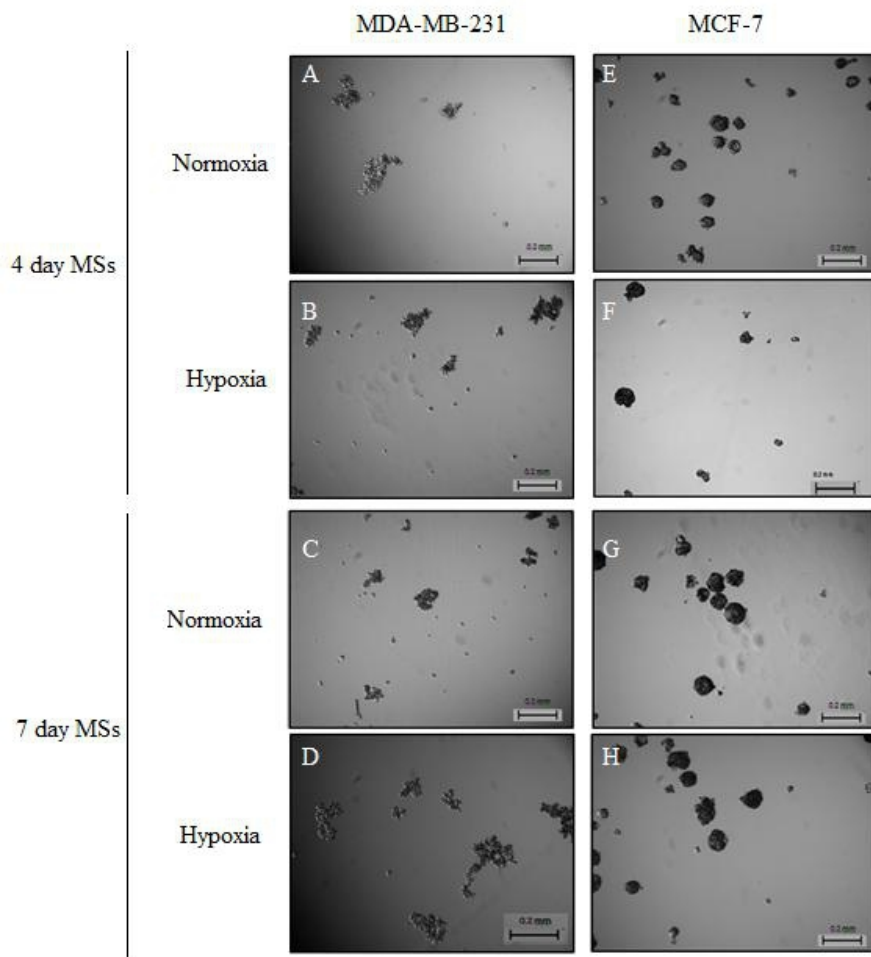


Figure 16A. Phase contrast microscopic imaging of MSs grown in normoxia or severe hypoxia at different developmental stages. (A-D) depict MDA-MB-231 MSs and (E-H) MCF-7 MS. In (A, C, E, and G) are the normoxic MS of the respective cell-line, in (B, D, F and H) are the hypoxic MSs.

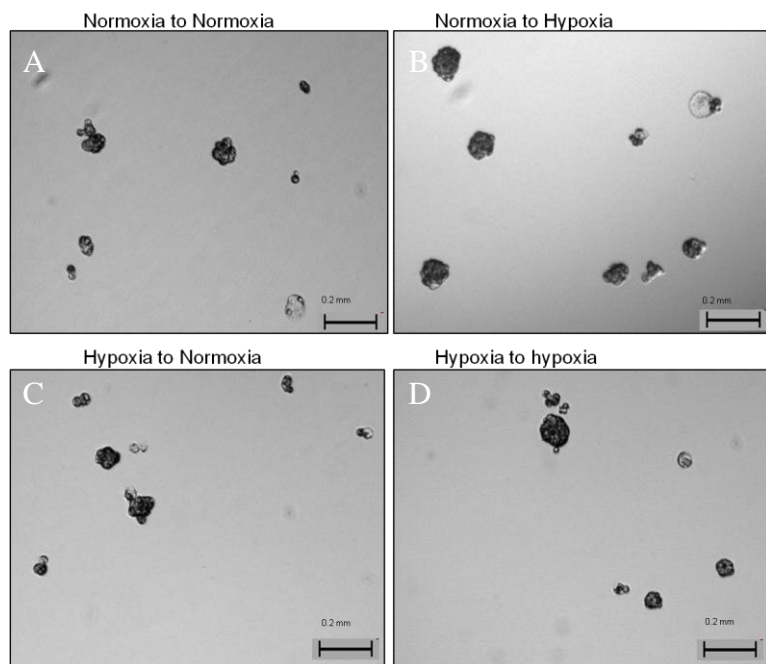


Figure 16B. Phase contrast microscopic imaging of 4 day MCF-7 MS after different pre-treatments and MS seeding conditions. (A) represents normoxic MSs issued from a normoxia treated adherent cell-line. (B) Depicts hypoxic MSs issued from an adherent culture pre-treated with normoxia. (C) Normoxic MSs obtained from hypoxia pre-treated parental cells are visible. (D) Shows MSs grown and pre-treated with hypoxia.

V. Results and discussion

A. Effect of hypoxia on the morphology of mammospheres

Phase-contrast microscopy imaging reveals the morphology of the MDA-MB-231 and MCF-7 MSs cultured in normoxic or severe hypoxic conditions at two different developmental stages, 4 and 7 days (Fig. 16A). The adherent culture is seeded at a density of 1000 cells/ml of medium in order to carry out the study of MS morphology.

The MSs formed by MDA-MB-231 cells appear to be quite loose and are very fragile. Their shape is random and the individual cells composing the sphere are well distinguished. These MSs can easily be dissociated, requiring any manipulations of these formations to be undertaken with a light touch. Based on repeated observations, the size of the normoxic spheres appears to be slightly smaller at developmental day 7 than at day 4. The hypoxic MSs, on the other hand, seem to moderately increase in size over the same time period. There is no particular difference that can be made between the normoxic and hypoxic MSs after 4 days. At developmental day 7 the hypoxic MSs have a considerably greater size than the normoxic MSs.

By contrast, MCF-7 MSs form dense regular spherical structures. The cells composing the spheres cannot be distinguished very easily; the cells seem to be tightly attached, giving the formation a more compact appearance than compared to the MSs formed by the MDA-MB-231 cell-line. The MCF-7 spheres are robust and are not easily dissociated. An increase in the size of the MSs can be observed over the course of time. In this cell line the MS morphology does not appear to be affected by the oxygen concentration as is the case in MDA-MB-231 MSs.

Additionally the impact of a 2 day pre-incubation in normoxia or hypoxia of the MCF-7 adherent cell cultures has been investigated. The microscopic imaging of such pre-treated parental cells subsequently cultured in MS forming conditions for a period of 4 days reveals that the size, shape and density of the MSs cultured in these 4 different combinations of oxygen conditions appear to be the same (Fig. 16B). No apparent effect of these pre-treatments on the morphology of the MCF-7 MSs can be observed whether they are cultured in normoxia or hypoxia.

These results indicate that the MSs formed by MDA-MB-231 and MCF-7 cells do not have the same response to hypoxia. Only the MDA-MB-231 MSs appear affected in their shape by the concentration of oxygen available. The reason for the differing response to hypoxia is not understood. It is obvious that this hypoxic effect could simply be cell line dependent. It could be due to proliferation differences in response to the oxygen environment. A recent paper by Harrison *et al.* evokes the importance of the presence or absence of ER- α expression on the CSC responsiveness to hypoxia (Harrison *et al.*, 2012). The authors demonstrated a HIF-1 α -dependent CSC increase in ER- α positive cell lines and cancers following hypoxic exposure, which was blocked by inhibition of oestrogen and Notch signalling. A contrasting decrease in CSC was seen in ER- α negative cell lines and cancers. Harrison's paper emits the hypothesis that ER- α positive cells, such as the MCF-7 cell-line, have altered self-renewal and increased dedifferentiation in response to hypoxia, whereas the ER- α negative cells, like the MDA-MB-231 cell-line, have altered proliferation pathways. Therefore, not only the proportion of CSCs contained in these formations but also the composition of their differentiated cells could be affected by the oxygen environment. Consequently one could consider that MSs could have a greater size, without affecting the number of spheres in the plate, if there is a higher proportion of proliferating cells or if proliferation is activated by hypoxia (e.g. in MDA-MB-231 cells). To confirm this hypothesis proliferation could be measured by analysing the abundance of Ki67, which is a well-known

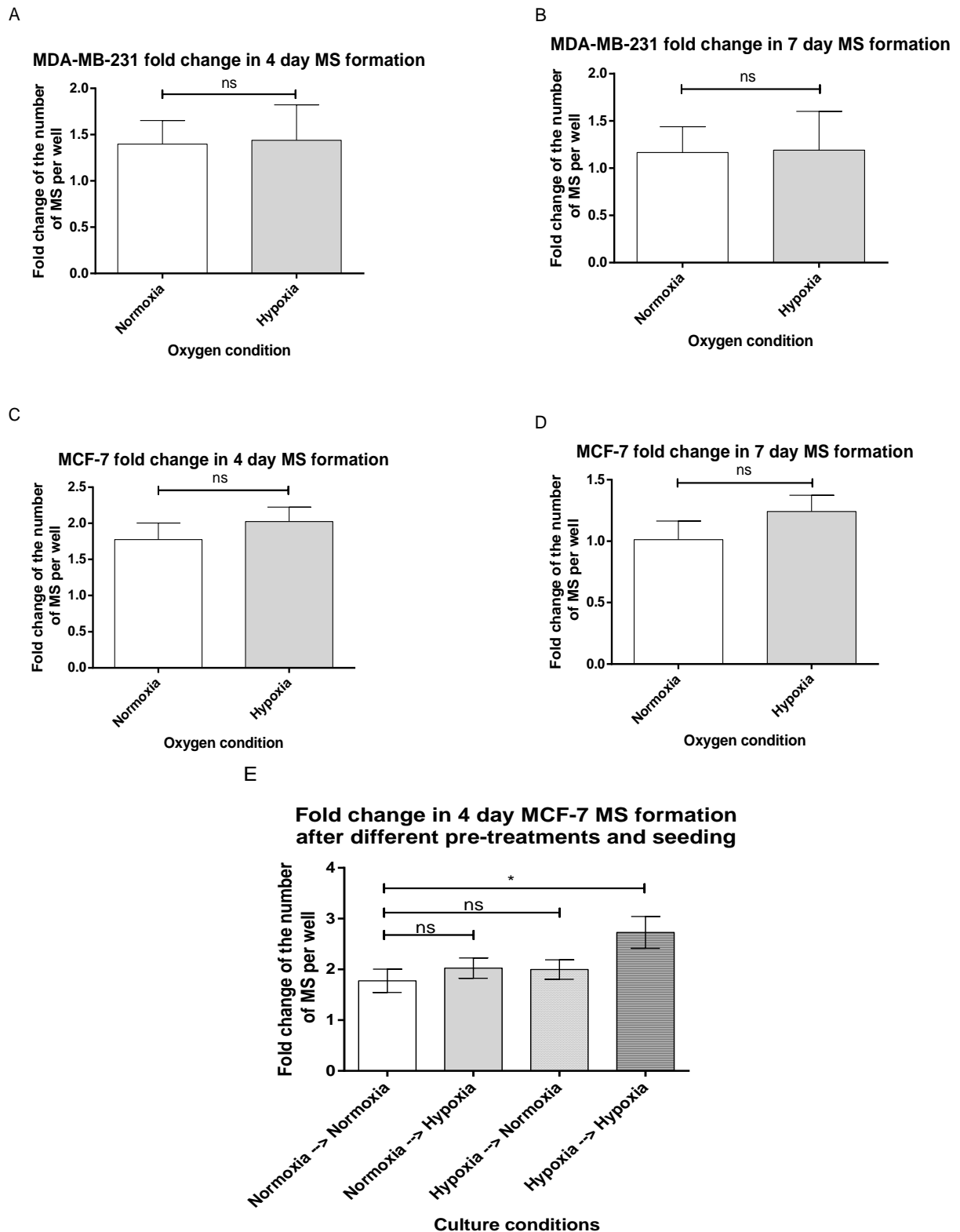


Figure 17. Fold change in MS formation in different oxygen concentrations. Comparisons are made to the strictly normoxic MS formations. (A and B) Represent the fold change in MDA-MB-231 MS formation and (B and C) the MCF-7 MS formation after respectively 4 and 7 days in culture. (E) Represents the 4 day MCF-7 MS fold change after pre-treating the adherent cell-line and then seeding the isolated cells with different combinations of oxygen concentration. The analyses are carried out using a two-way ANOVA with Bonferroni posttests. (* = $P < 0.05$ and ns = non significant)

proliferation marker. A verification that the MCF-7 cell line we use does indeed express ER- α and that the MDA-MB-231 cell line does not was carried out and confirmed by a colleague.

Another possibility for the increased size of hypoxic MDA-MB-231 MSs at 7 days is a stronger interaction between cells, thus reducing the number of cells that get detached from the formation. Indeed it is known that hypoxia, through the intermediate of HIFs, can affect proteins involved in cellular adhesion and can modify the ECM (Harris, 2002). CD44 is a glycoprotein that is involved in cellular interactions and that is differently expressed in response to hypoxia. The cell-cell and cell-ECM interactions mediated through CD44 and HA can also modify the cancer cells' survival mechanisms. This could contribute to an increased MS size due to a larger proportion of cells surviving in the hypoxic conditions (Bourguignon et al., 2009; Toole, 2004). The expression of CD44 has been investigated during this study and will be detailed later on (no significant difference was observed, however). An alteration of the ECM due to a different abundance of HA could also provoke a change in the morphology of the MSs. A dosage to determine the quantity of HA released into the medium and present in the cellular fraction has also been carried out throughout this study and will be discussed later (an increased HA production during hypoxia in MDA-MB-231 but not in MCF-7 MSs was confirmed).

B. Effect of different oxygen concentrations on mammosphere formation

The number of MSs formed by both cell lines seeded in non-adherent conditions at a density of 1000 cells/ml is a method of representing early stem cell or progenitor activity. It indicates how well the CSCs issued from the adherent cell cultures survive in suspension. It is important to note that this method does not provide any information on the self-renewal capacity of the stem cells. By seeding the cells in different oxygen conditions it may be possible to determine which oxygen concentration is more favourable to MS formation. The results depicted in Fig. 17 (A-D) represent the fold induction of MS formation, when compared to one chosen normoxic replicate at developmental days 4 and 7. An unpaired t test was utilised in order to determine whether the fold inductions between normoxic and hypoxic MSs are significantly different.

In MDA-MB-231 MSs there is no observable difference between the formations of MS depending on whether the cells were seeded in normoxic or hypoxic conditions at developmental days 4 and 7. However, counting MDA-MB-231 MSs is delicate work as the formations are extremely fragile. During the transfers from the 6-well plates to the 24-well plates it is possible that the spheres are broken, biasing the number of MSs counted. To avoid the problems of mechanical handling it was attempted to seed the spheres directly onto PBS-agarose 1% coated 24-well plates. However, this method comes with the disadvantage that the MSs are found concentrated in the middle, making it difficult to single out the individual MSs.

The MSs formed by MCF-7 cells are more resistant, thus transferring them from one well to another does not cause a significant amount of manipulation induced dissociation. Proportionally to normoxia the initiation of MS formation by MCF-7 cells does not appear to be affected by hypoxia after 4 days in normoxic or hypoxic conditions although there is an insignificant tendency to an increase in hypoxic MS formation. This non-significant difference between MS growth in normoxia and hypoxia is maintained throughout both developmental stages.

Due to the technical difficulties in studying the number of MDA-MB-231 MSs and the considerable financial aspect of each seeding, it was deemed appropriate to further inquire the effects of hypoxia on MS formation in solely the MCF-7 cell-line (Fig. 17E). In these studies on MCF-7 cells the influence of pre-treating the parental cell line with different oxygen

conditions before seeding the cells in normoxic or hypoxic MS forming conditions was explored. These MSs were then cultured for 4 days in the specific oxygen condition. As previously mentioned the adherent cultures pre-treated in normoxia and then seeded in normoxia (normoxia to normoxia) or hypoxia (normoxia to hypoxia) do not bear a significant difference in fold change of MS formation. Pre-treatment of the MCF-7 parental line in hypoxia does not significantly influence the fold change in the number of MSs formed in normoxia (hypoxia to normoxia) although a slight tendency to increase can be observed. The adherent cells pre-treated in hypoxia and then seeded into a hypoxic MS forming environment (hypoxia to hypoxia) have a significantly increased number of MSs compared to the normoxic MSs.

In summary, only when both the pre-treatment and the seeding conditions are at a low oxygen concentration is a significant increase in MCF-7 (but not in MDA-MB-231) MS formation observed when compared to the strictly normoxic MSs. This phenomenon has not been described in the literature yet. The results published by Harrison *et al.* (increase in MS formation in the hypoxic to normoxic condition) have not been reproduced in this study but it must be noted that the seeding conditions are not entirely equal. For instance, there are differences in the medium used and in the incubation durations.

The increased MS formation in the hypoxic pre-treated MSs cultured in hypoxia could be due to an increase in the proportion of CSC present in the total adherent population of cells. It could also be due to the fact that hypoxia favours the initiation of MS formation. The fact that no increase in MS formation occurs in the normoxic MSs issued from a hypoxic pre-incubation indicates that the presence of a hypoxic environment in the adherent culture on its own does not favourably affect the cellular processes of CSCs seeded in normoxia. But it mainly indicates that it probably isn't the proportion of MS that is modified by this pre-treatment. If this had been the case an increase in MS formation should have been observed in the hypoxic to normoxic conditions. Thus, the hypoxic treatment prior to anchorage independent growth may in a way condition the CSCs to better survive in the hypoxic environment. The hypoxic environment can thus be favourable under the condition that the CSCs have already been subjected to these low oxygen conditions. In a similar fashion, Louie *et al.* have shown that repetitive cycles of hypoxia and re-oxygenation but not short bouts of hypoxia are able to increase the proportion of CSCs in two BC cell lines, MDA-MB-231 (where we did not test the effect of hypoxic pre-incubation) and BCM2 (Louie *et al.*, 2010).

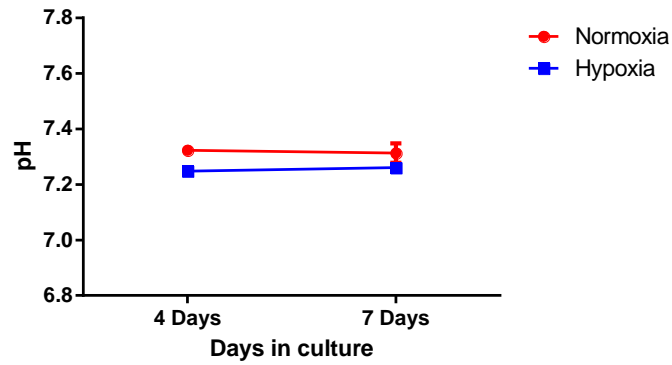
Thus, the particular effects of different exposures to hypoxia may modulate the CSC subpopulation in human BC. It would be useful to see whether the hypoxic effect we observed is MCF-7 cell line specific or whether it occurs in other BC cell lines. As Harrison *et al.* evoke that the response to hypoxia is mediated by the ER- α status it would be interesting to inspect this too. To do so one could use multiple cell lines from ER- α positive and ER- α negative cells that would be seeded in the various oxygen concentration combinations. To determine the implication of the ER- α status on the response to hypoxia, the receptor could be knocked down in ER- α positive cells, and, *vice versa*, the ER- α negative cell lines could be transfected with the gene. Secondary sphere formation could also provide additional information, such as whether it is the self-renewal processes that are affected by hypoxia. Further investigation aiming to uncover the mechanisms underlying the increased potency to form MS in these strictly hypoxic conditions would constitute compelling work that could be performed in the future.

C. The effect of hypoxia on the pH of the mammosphere culture medium

The pH of the media collected from MDA-MB-231 and MCF-7 MSs was measured at day 4 and 7 of their development.

A

pH measurement of MDA-MB-231 MS medium after culture in normoxic or hypoxic conditions



B

pH measurement of MCF-7 MS medium after culture in normoxic or hypoxic conditions

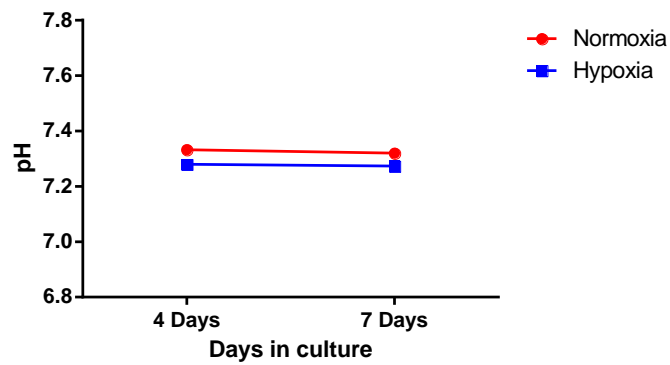


Figure 18. pH values of the medium in which the MSs were cultured. (A) Represents the pH of the MS medium issued from MDA-MB-231 MS cultured in normoxia or severe hypoxia for 4 and 7 days. (B) Shows the pH values of the medium of MCF-7 MSs cultured in normoxia or hypoxia for 4 and 7 days.

The MSs formed by MDA-MB-231 do not show any specific change in pH throughout the development of the spheres, in both normoxic and hypoxic conditions (Fig. 18). The pH remains within the neutral range. The curve representing the pH of the hypoxic MSs is lower than that of the normoxic MS and this difference is significant. This is valid for both developmental stages. The measured pH is considered as neutral in both conditions. Almost identical observations are made with MCF-7 MS cultures.

These results indicate that either hypoxia itself or the hypoxic MSs induce a slight reduction of pH of the medium, although this reduction is significant only for the MDA-MB-231 cell line. The pH of the medium is contained in the neutral pH range for both cell lines and in both conditions.

Tumour masses usually have a lower pH than normal non malignant tissue, and cells exposed to hypoxia can also have a low pH due to the altered metabolism (Al Okail, 2010; Tannock, Rotin, & Hot, 1989). Thus it's not surprising to see that the pH diminishes in hypoxia. Due to the fact that the medium contains buffers that stabilise the pH a titration of the medium would have had to be performed in order to determine to what extent the hypoxic MSs manage to induce a reduction of pH of the medium. The reduced pH of the medium may indicate that the cells are exchanging protons in order to reduce extent of intracellular acidosis, which is common in cancer cells but can be exasperated by hypoxia. Further studies could be performed in order to determine whether the cells are indeed in acidosis. One of the molecules involved in acidosis that is highly produced in cancer cells through the altered metabolism is lactate. Lactate is involved in many cellular processes and can impact cancer progression. Promotion of metastasis can be mediated through lactate induced HA secretion by tumour associated fibroblasts, cellular migration can be stimulated, and the anti-oxidative properties of lactate can offer radio-resistance to the cancer cells (Hirschhaeuser et al., 2011). The expression of many mediators of HA metabolism can be stimulated by lactate. This indicates that a dosage of lactate could offer some insight to any changes in HA metabolism that could arise in cancer cells, especially when exposed to hypoxia.

D. Effect of different oxygen concentrations on the metabolism of hyaluronan

1. Effect of hypoxia on the production and secretion of hyaluronan

The effect of the oxygen environment is studied at two developmental stages in MDA-MB-231 MSs and MCF-7 MSs. The quantity of HA present in the cellular fraction is measured as well as that released into the medium over a 24 h period. The HA measured in the cellular fraction corresponds to that contained within the cell, but also that bound to the cell. The statistical analyses are only possible for the MDA-MB-231 MSs as there is at least one replicate missing from the MCF-7 MSs (Fig. 19).

The production of HA in the cellular fraction of MDA-MB-231 MSs does not appear to vary over the course of time in normoxia. In hypoxia the production of HA also appears to remain consistent over the course of time. At day 4 there is a higher HA production in hypoxic MSs compared to those in normoxia. At this developmental stage the difference is significant. At 7 days the hypoxic MSs appear do not present a significant difference in the production of HA compared to the HA production normoxia, but the variability is rather high at this developmental stage making it difficult to draw any specific conclusions.

The release of HA into the medium by MDA-MB-231 in both normoxia and hypoxia appears to increase after 7 days in culture. The secretion of HA in normoxic and hypoxic MSs at developmental day 4 and 7 appears to bear a tendency to be higher in the medium of the hypoxic MSs.

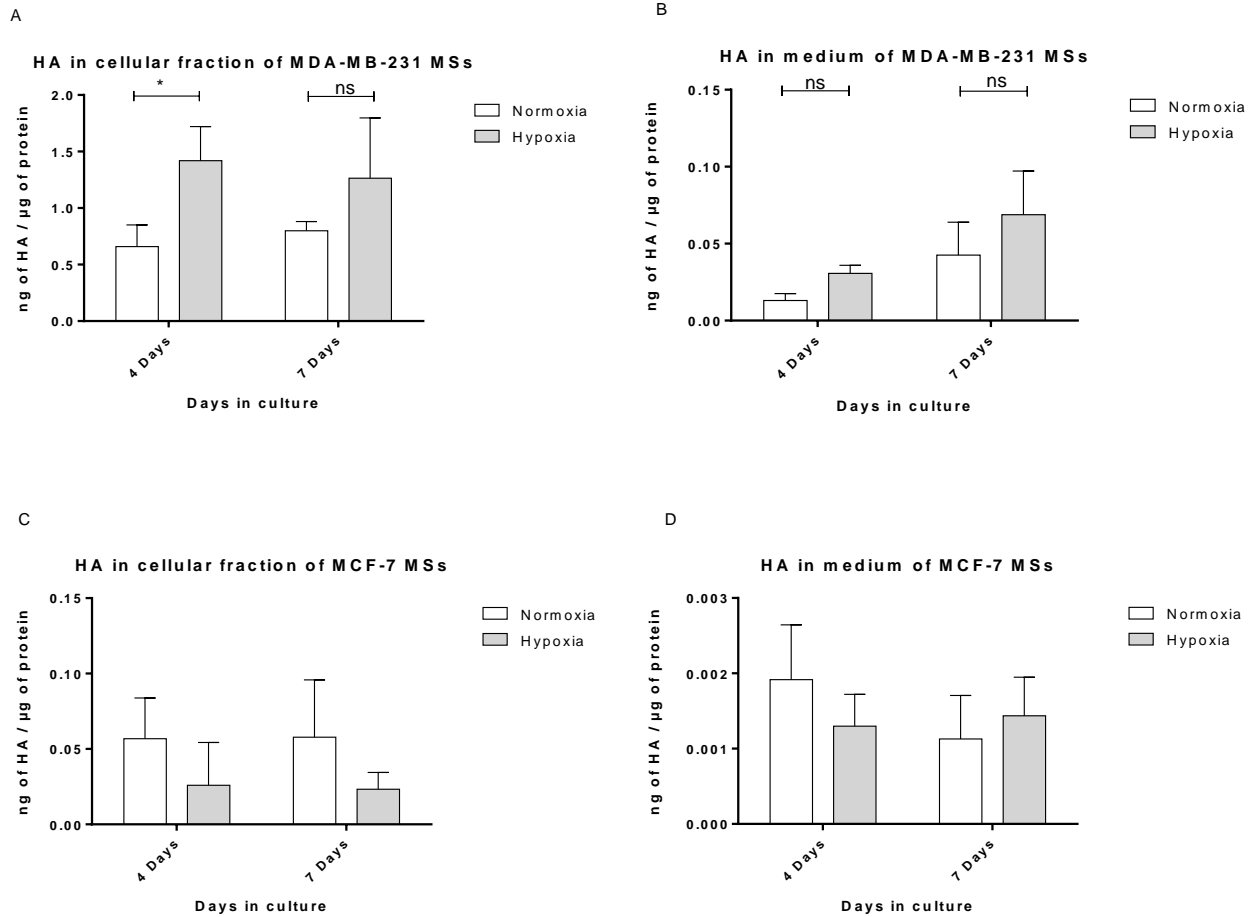


Figure 19. Dosage of the HA in the cellular fractions and in the medium of MS cultured in different oxygen conditions and at different developmental stages. (A and C) represents the quantity of HA produced in 24h in the cellular fraction of MS at two developmental stages. (B and D) depict the quantity of HA released into the medium during 24 h by normoxic and hypoxic MSs at two developmental stages. The data collected for MDA-MB-231 (A and B) MSs is issued from 3 separate replicates, and that of the MCF-7 MSs (C and D) is issued from 2 replicates. Statistical analysis will only be possible for the MDA-MB-231 cell-line due to missing replicates for the MCF-7 cell-line. The MDA-MB-231 data is analysed using a two-way ANOVA with a Bonferroni posttest. The quantity of HA is normalised to the quantity of proteins of each sample. (* = $p < 0.05$)

Regarding the MCF-7 cell line, the production of HA in the cellular fraction of the MSs appears to remain unvaried over time in normoxia and hypoxia. The production of HA seems to be lower in hypoxic MS after 4 days in culture than that of normoxic MSs. This tendency to a reduced quantity of HA seems to be maintained at developmental day 7.

The release of HA into the medium by MCF-7 MS appears to decrease over time in normoxic conditions, whereas it appears to remain unvaried in the hypoxic conditions. At developmental day 4 the release of HA does not show any particular difference between the normoxic and hypoxic MSs. At developmental day 7 no difference in HA release can be made either between normoxic and hypoxic MSs.

In summary, hypoxia has a moderately stimulating effect on the production of HA and on its release into the medium in MDA-MB-231 MS cells but not in MCF-7 MSs. This increased synthesis could be caused by an increased expression of the HA synthesising enzymes, such as HAS2 and HAS3. The expression of the genes coding for HAS2 and HAS3 are studied at the transcriptional level and will be detailed in the coming paragraphs. Previous studies conducted in the laboratory indicate that HAS1 is not expressed in MDA-MB-231 and MCF-7 MSs, thus the study of this protein is excluded from this Master's Thesis. In the MDA-MB-231 cell line the different levels of production and release of HA in hypoxia could also be due to differences in the activity of the HAS enzymes that could possibly be induced by hypoxia. The study of the activity of these enzymes was not achievable during this particular research but could be an avenue worth exploring in the future. A higher number of replicates would be of interest as the variability is quite high. The fact that the quantity of HA appears to have a tendency to increase in hypoxia could also be caused by a slower turnover rate, meaning that the HYALs could be less active or their expression diminished.

It seems that hypoxia has an opposing effect (or at least an opposing trend) on the production and release of HA in the MCF-7 MSs than that seen in MDA-MB-231 MSs. The release of HA into the medium does not appear to be affected by the oxygen concentration. In MCF-7 MSs hypoxia does appear to affect the production of HA in the cellular fraction as they reveal a slight tendency to have a reduced secretion of HA compared to that of the normoxic MS. Due to insufficient data it is not possible to tell whether the decrease in severe hypoxic conditions is merely a tendency or whether it represents an actual significant decrease. This reduced production of HA could be caused by a reduction in the expression of HAS proteins. The transcriptional expression has been studied and will be described later on. It is also possible that the decrease is a consequence of a reduced activity of the enzyme, which may be caused by the hypoxic conditions. The tendency to a reduction in HA quantities may also be caused by different expression levels and activities of HYALs.

Looking into the cellular mechanisms leading to the fact that HA production in these two cell lines is differently affected by hypoxia could also amount to some interesting work. Evidently it would also be fascinating to determine whether the abundance of the different HA fragment sizes is altered by the oxygen environment. As mentioned in the introduction, the physiological action that HA can have on the physiology of a cell and how it can contribute to cancer biology is dependent on the size of the fragments. Studying this could be achieved by dosing the quantity of HA after having separated the fragments of HA using size-exclusion chromatography.

2. CD44 expression in different oxygen conditions

The effect of the oxygen environment on the expression of CD44, a stem cell marker and also the main receptor for HA, is studied in MDA-MB-231 and MCF-7 MSs at two developmental stages. Four and three replicates have been gathered for MDA-MB-231 and MCF-7 MS RT-qPCR analyses, respectively (Fig. 20).

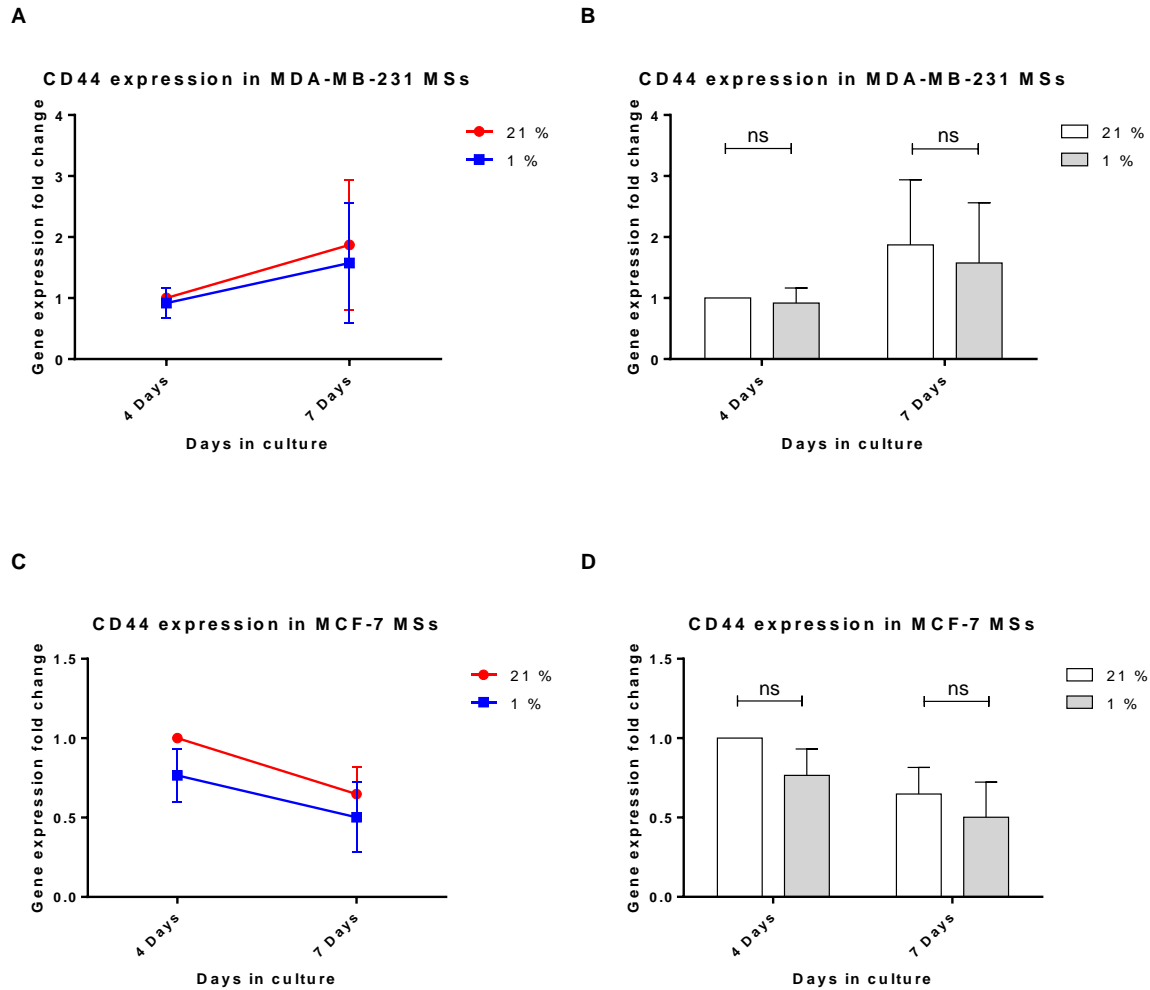


Figure 20. Fold change in transcriptional expression of CD44 in MS cultured for 4 and 7 days in normoxia or hypoxia. (A and B) represent MDA-MB-231 cell-lines, values were obtained from 4 individual replicates. (C and D) represent MCF-7 cell-lines, for which 3 replicates were used. The values are normalised to actin expression. The statistical analyses were carried using a two-way ANOVA with Bonferroni posttests. The analysis is performed on the $\Delta\Delta\text{CT}$ and the graphs show the fold change ($2^{-\Delta\Delta\text{CT}}$). (ns = non significant).

In MDA-MB-231 MSs, the expression of CD44, assessed through RT-qPCR, shows a tendency to increase over time, from 4 to 7 days in culture. After 4 days in culture the expression of CD44 does not reveal any differences between the two oxygen conditions. After 7 days of culture no difference in expression of the HA receptor can be detected either. The variability at developmental day 7 is considerable, evoking the fact that additional replicates should be added in order to better assess the variation in transcriptional CD44 expression between both conditions.

RT-qPCR measurements of CD44 expression in MCF-7 MSs seem to reveal a diminishment in expression in both oxygen conditions over time. At developmental day 4 the expression of CD44 does not show any statistical difference between both oxygen conditions; this is also the case at developmental day 7 although there does seem to be a tendency to a lesser expression in the hypoxic MSs compared to the normoxic MSs. Again the variability is rather high.

The fact that no significant difference in the expression of CD44 can be detected between the normoxic and hypoxic MSs is somewhat surprising as a publication in literature evokes that hypoxia via the intermediate of HIF up-regulates CD44 expression (Krishnamachary et al., 2012). There are a couple of possibilities that could explain this discrepancy. First of all CD44 expression seems to evolve throughout time. For example in the MCF-7 cell line a tendency to a diminishment in CD44 expression can be seen throughout the development. It might be that any effects of the oxygen expression on CD44 in MSs could have occurred prior to day 4. Thus studying the effect that normoxia and hypoxia could have on CD44 expression at an earlier stage, closer to the initiation of the MSs, would be interesting. This exact phenomenon has been observed in unpublished studies performed by a colleague working in the same MS forming conditions in the laboratory. RT-qPCR performed on MCF-7 MS at developmental day 2 reveal a 5-fold increase in CD44 expression in the hypoxic MSs (identical sense and anti-sense primers were used for both studies).

The RT-qPCR study carried out on the expression of CD44 is performed using primers that have a ubiquitous recognition of CD44 isoforms. Studying whether the oxygen concentration has an influence on specific splice variants could be an avenue worth exploring, especially as there is a link between poor patient outcome and a higher abundance of the alternative splice forms such as CD44v3 and v6 in comparison to the standard CD44 splice form. With the chosen method of CD44 expression throughout this particular study such differences are not detectable.

3. HAS2 expression in different oxygen conditions

The expression of HAS2 has been analysed in MSs cultured in normoxia and severe hypoxia at 2 different developmental stages by RT-qPCR (Fig. 21).

The focus will first be set on MDA-MB-231 MSs. There is a significant increase in HAS2 expression over the course of time in the hypoxic MSs when compared to that of the normoxic MSs. Both of the studied variables, notably the oxygen concentration and the days in culture, have a significant effect on the expression of HAS2. At developmental day 4 there is a non-significant tendency to an increase in mRNA for HAS2 in the hypoxic condition. At developmental day 7 this hypoxic tendency to an elevated expression becomes statistically significant when compared to the normoxic condition.

The expression of HAS2 in MCF-7 MSs does not appear to evolve between 4 and 7 days after seeding, in both the normoxic and hypoxic environment. At developmental day 4 and 7 there is no particular difference in expression that can be made evident between the normoxic and hypoxic seeding conditions. However, variability is high and the number of experiments ($n = 3$) is limited.

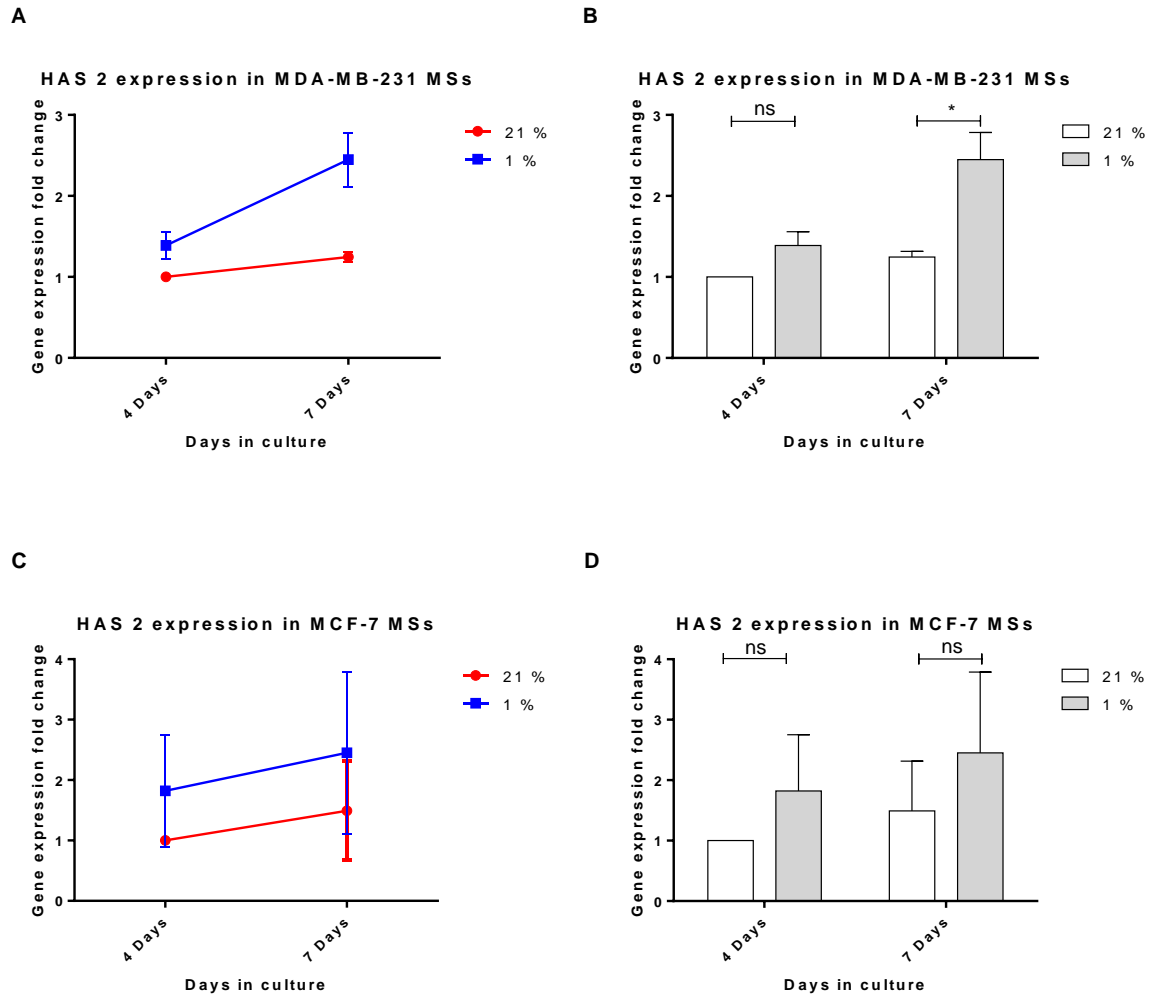


Figure 21. Fold change in transcriptional expression of HAS2 in MS cultured for 4 and 7 days in normoxia or hypoxia. (A and B) represent MDA-MB-231 cell-lines, values were obtained from 4 individual replicates. (C and D) represent MCF-7 cell-lines, for which 3 replicates were used. The values are normalised to actin expression. The statistical analyses were carried using a two-way ANOVA with Bonferroni posttests. The analysis is performed on the ΔCT and the graphs show the fold change ($2^{-\Delta\Delta\text{CT}}$). (ns = non significant and * = $p < 0.05$).

The fact that there is an increase in HAS2 expression in hypoxic MDA-MB-231 MSs is a captivating find as HAS2 is a pertinent factor in tumour biology and has been found to be involved in potent cancer progression pathways. Recently HAS2 has been described as an essential factor for bone metastasis in breast cancer (Okuda et al., 2012). The up-regulation of this HA synthesizing enzyme in CSCs is associated with an increased risk of metastasis and an enhanced ability of the cells to adhere to immortalized mouse bone microvascular endothelial cells (mBMEC) *in vitro*. These two factors are necessary for metastasis. In their studies Okuda *et al.* used a highly metastatic MDA-MB-231 cell-line derivative known as 231-BoM. They compared the metastatic abilities of this cell line to that of the conventional MDA-MB-231 cell lines. They saw that the highly metastatic 231-BoM cell line had a high expression of HAS2 compared to that of the MDA-MB-231 cell line. They showed that HAS2 was the key element to the metastatic ability. Consequently, observing such an increase in HAS2 in the hypoxic MSs of our study reinforces the statements that hypoxia can favour cancer progression. This needs of course to be verified. One could investigate the invasive abilities of the cells composing these hypoxic MSs by seeding the dissociated MSs in Transwell inserts placed over a monolayer of mBMEC. Adherence could be examined by seeding the dissociated MSs issued from a hypoxic environment on a monolayer of mBMEC. One could also envisage examining whether hypoxia affects ability to survive *in vivo*, by injecting cells from the hypoxic MS into nude mice. It has already been shown that inhibition of HAS2 *in vivo* can totally inhibit the progression of primary breast cancer (Udabage et al., 2005). These results could further provide proof of the importance of HAS2 in the malignant progression of BC.

As one of the only established links to the differing responses to the oxygen environment within breast CSCs is mediated through the ER- α status, it could be interesting to investigate whether this status can also be tied to the high expression of HAS2 in response to hypoxia.

4. HAS3 expression in different oxygen conditions

RT-qPCR analyses aims to detect any possible differences in the transcriptional expression of HAS3, another HA synthesising enzyme, that could be caused by a hypoxic environment (Fig. 22).

MDA-MB-231 MSs show a considerable decrease in HAS3 expression at 7 days compared to the level of expression at 4 days, but only in the normoxic condition. The HAS3 expression in hypoxic MSs does not appear to be affected in such a way. At developmental day 4 the level in HAS3 expression seems to be equal between both normoxic and hypoxic conditions. After 7 days of growing in the specific oxygen environment there appears to be a higher level of expression in the hypoxic MSs, but this is not statistically validated and the variability is quite high. Increasing the number of replicates would be of interest.

The expression of HAS3 in MCF-7 MSs appears to remain rather constant over the course of the chosen times. This is valid for the normoxic and hypoxic MSs. The expression of HAS3 at developmental day 4 appears to be higher in the MSs cultured in the low oxygen conditions compared to that of the MS cultured in normoxia. This tendency is not statistically validated. At developmental day 7, the variability in the hypoxic MSs is quite high, making it impossible to draw conclusions. Additional replicates are needed in order to allow an optimal analysis of the expression of HAS3 expression in the two different oxygen concentrations.

HAS3 does not show any significant differences in expression in response to hypoxia in this experimental model, for both MDA-MB-231 and MCF-7 cell lines. In MCF-7 MS there does seem to be a tendency of a higher expression of HAS3 in MS cultured in hypoxic conditions compared to the normoxic MS. Increasing the number of replicates could reveal

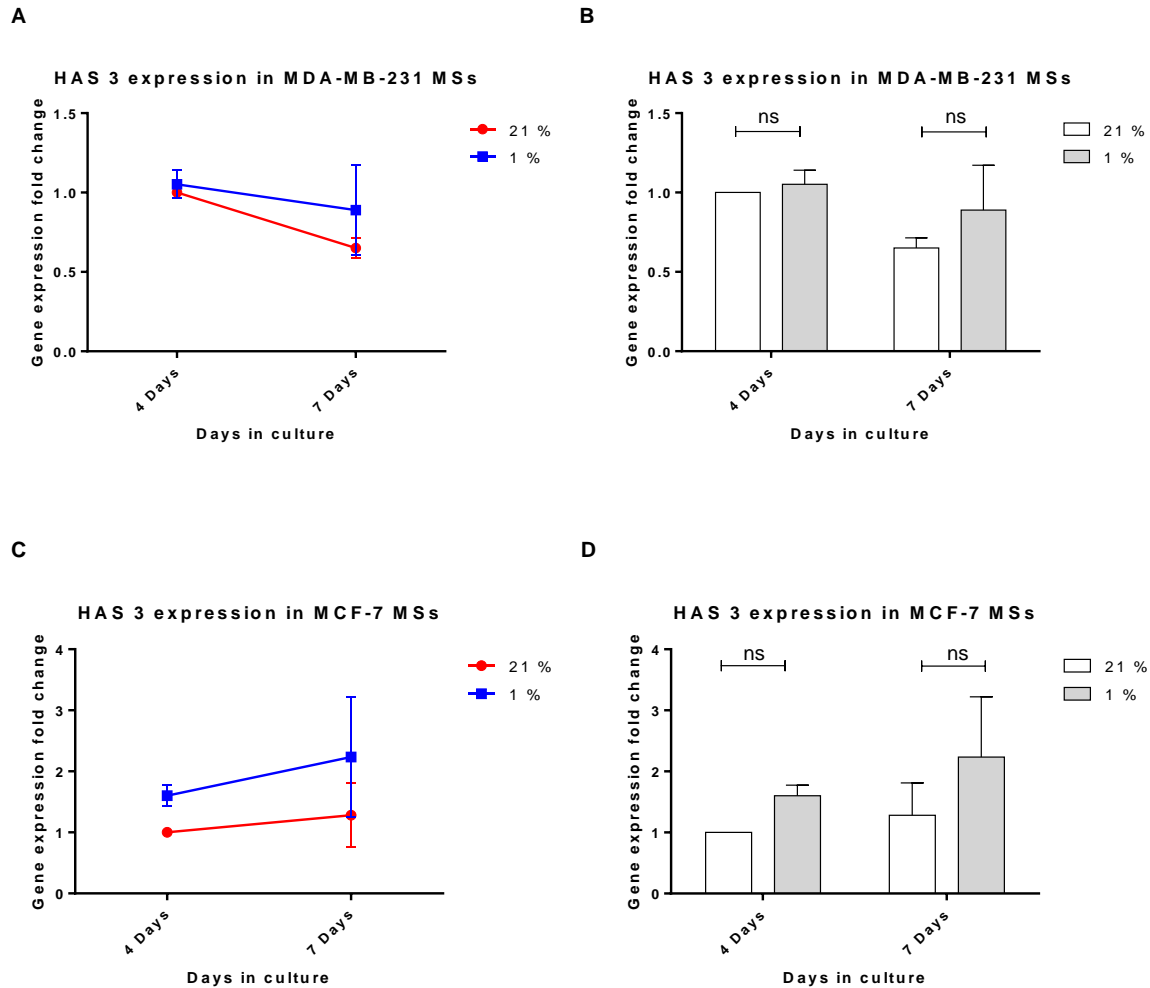


Figure 22. Fold change in transcriptional expression of HAS3 in MS cultured for 4 and 7 days in normoxia or hypoxia. (A and B) represent MDA-MB-231 cell-lines, values were obtained from 4 individual replicates. (C and D) represent MCF-7 cell-lines, for which 3 replicates were used. The values are normalised to actin expression. The statistical analyses were carried using a two-way ANOVA with Bonferroni posttests. The analysis is performed on the ΔCT and the graphs show the fold change ($2^{-\Delta\text{CT}}$). (ns = non significant).

whether this is a true increase or not. In BC models an increase in HAS3 has not been reported yet. In colon carcinomas, on the other hand, an increase in HAS3 and the production of HA have been seen to contribute to carcinoma growth and progression (Bullard et al., 2003). Further studies on the expression of this protein could thus provide researchers with vital information, especially for the development of novel treatments. It has already been established *in vivo* that inhibition of HAS2 can limit cancer progression, thus it wouldn't be implausible to envisage inhibition of HAS3 having a similar effect on the malignant development of tumours.

5. HYAL1 expression in different oxygen conditions

The expression of HYAL1 in MDA-MB-231 MSs at 4 and 7 days cultured in different oxygen concentrations, namely normoxia and severe hypoxia, was studied at the protein level by Western Blot analyses. Due to technical difficulties the sample size is small. Nonetheless, the graphs do give an indication of how HYAL1 is expressed in normoxic and hypoxic conditions (Fig. 23).

In MDA-MB-231 MS the expression of HYAL1 doesn't seem to vary between developmental day 4 and 7 in the normoxic MSs or in the hypoxic MSs. At developmental day 4 there doesn't appear to be any difference in protein expression between normoxia and hypoxia. After 7 days in normoxic or hypoxic conditions no particular difference in HYAL1 expression can be seen either.

The expression of this HA degrading enzyme doesn't appear to develop in MSs formed from MCF-7 cell lines over 4 and 7 days whether the cells are exposed to normoxia or hypoxia. When comparing the expression of HYAL1 in normoxic and hypoxic MSs at developmental day 4 there is no indication of a difference in expression. At developmental day 7 the expression of HYAL1 doesn't appear to be affected by the oxygen concentration.

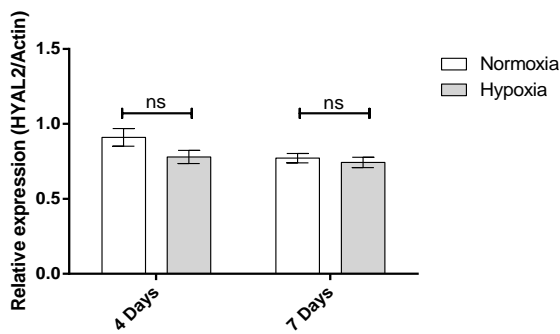
These results give a preliminary indication that the expression of HYAL1 doesn't appear to vary in function of the oxygen concentration in which the MS were cultured. It doesn't seem as if in this particular model either HIFs or hypoxia induced altered metabolism can change the level of expression of HYAL1 in MSs exposed to these different oxygen concentrations. It would be interesting to ascertain whether the activity of the enzyme shows any variations after having been exposed to normoxia or severe hypoxia. There exist different classes of HYAL inhibitors that have a crucial role of ensuring that HA degradation is fulfilled in a balanced manner. It has been observed that in cancer patients the classes of HYAL inhibitors can be expressed in different proportions and can give insight whether the patient will have a good outcome or not (Stern, 2005, 2008a). Thus in the future one could investigate whether the activity of HYAL1 is altered by hypoxia, and if that is the case the proportion of HYAL inhibitors could be examined too. Another avenue that could be explored is to see whether hypoxia induces the expression of different HYAL1 isoforms in a breast CSC model. In tumours an elevated expression of the wild-type HYAL1 isoform is associated with an increased amount of angiogenic inducing fragments. In lung cancer the abundance of these splice variants has been studied and found to influence patient outcome (de Sá et al., 2012).

6. HYAL2 expression in different oxygen conditions

The effect of the oxygen environment, normoxia compared to severe hypoxia, on HYAL2 expression in MSs formed by MDA-MB-231 and MCF-7 cells has equally been studied. Two different assays were conducted for this particular study. The western blot analysis aims to uncover any differences in the level of expression of HYAL2 that could

A

Effect of the oxygen condition on
HYAL2 expression in MDA-MB-231 MS



B

Effect of the oxygen condition on
HYAL2 expression in MCF-7 MS

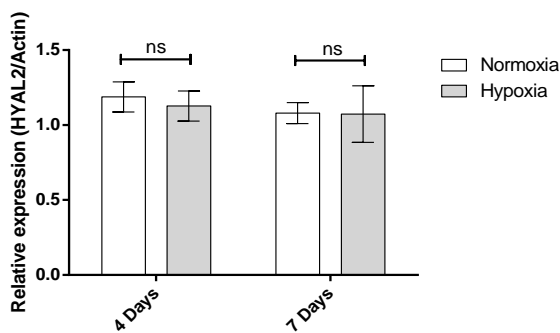
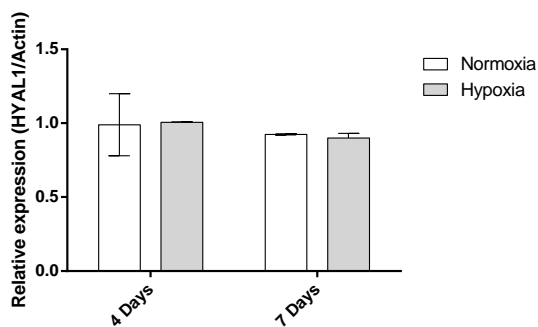


Figure 23. Protein expression of HYAL1 in MS cultured in normoxia and hypoxia. (A) MDA-MB-231 MSs cultured for 4 and 7 days in normoxia and hypoxia. The values are issued from 2 individual replicates. (B) MCF-7 MS cultured in normoxia and hypoxia for 4 and 7 days. The values are issued from 2 individual replicates. Statistical analysis is not possible due to the small sample size of both cell-lines.

A

Effect of the oxygen condition on
HYAL1 expression in MDAMB-231 MS



B

Effect of the oxygen condition on
HYAL1 expression in MCF-7 MS

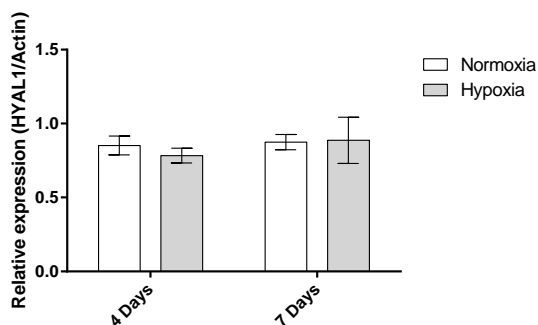


Figure 24. Protein expression of HYAL2 in MS cultured in normoxia and hypoxia. (A) MDA-MB-231 MSs cultured for 4 and 7 days in normoxia and hypoxia. (B) MCF-7 MS cultured in normoxia and hypoxia for 4 and 7 days. Statistical analysis is not possible due to the small sample size.

occur in response to a hypoxic environment (Fig. 24). An immunofluorescent staining aims to reveal the cellular localisation of the enzyme as well as its level of expression (but this level of expression is not quantifiable).

The western blot results on HYAL2 expression levels in MDA-MB-231 MSs will be described first. The expression of the HA degrading enzyme appears to have a slight tendency to decrease from day 4 to developmental day 7 in normoxic conditions. In hypoxic conditions the expression of HYAL2 appears to be consistent through the two stages of development. When comparing the levels of expression in normoxic and hypoxic conditions at day 4, a tendency to a reduction can be seen but the analysis reveals that this reduction is not significant. At developmental day 7 the expression level of HYAL2 seems to be equal between both normoxic and hypoxic MSs. The immunofluorescent staining of HYAL2 doesn't supply us with any consistent results, making it impossible to analyse the results acquired from the confocal imaging. This could be a consequence of the fact that the fluorescence emitted by the staining of HYAL2 is very faint and requires an amplification of the PMT which is not ideal as it decreases the signal to noise ratio.

The expression of HYAL2 measured in MCF-7 MSs after exposure to normoxia or severe hypoxia will first be characterised based on the western blot analysis and afterwards by the immunofluorescent staining. The expression of HYAL2 doesn't appear to differ over the course of the chosen time lapse both in normoxia and hypoxia. The expression at day 4 of the MSs culture doesn't show any difference in expression of the protein of interest between normoxic and hypoxic MSs. At day 7 there is equally no significant difference that can be observed. As for the MDA-MB-231 MSs, the immunofluorescent staining for HYAL2 is particularly faint in the MCF-7 MSs too. The same problems encountered for the analysis of the staining of HYAL2 in MDA-MB-231 MSs is valid for the MCF-7 MSs, making it impossible to reach any conclusions.

In summary, the results of the analysis of HYAL2 expression, although partial and not particularly supported by confocal analysis, seem to indicate that this HA degrading enzyme does not play a major role in the development of MS under hypoxic vs normoxic conditions. In fact, there is no obvious change in either HYAL1 or HYAL2 under the different conditions tested.

VI. Conclusion

The collected data shows that MDA-MB-231 and MCF-7 MSs, which are a model of CSCs/TICs, respond to low oxygen conditions in quite different ways. The cells issued from the more aggressive ER- α negative cell line, MDA-MB-231, acquire changes in MS morphology and in HA metabolism, whereas the less aggressive ER- α positive MCF-7 cell line displays accelerated MS formation and growth.

MCF-7 cells

MSs formed by MCF-7 cell lines didn't show any significant variations in how they metabolise HA after exposure to severe hypoxia. It must be pointed out that the number of replicates for this cell line is quite low, and due to many instances of high variability between the samples the establishment of conclusions must be done with much precaution. The RT-qPCR and HA dosage do show that the metabolism of HA could be modified by hypoxia, as both HAS proteins have a tendency to increase after seeding in hypoxic conditions. On the other hand the dosage of HA shows a slight tendency to a diminished quantity of HA in the cellular fraction. This inconsistency between transcript expression and HA dosage could have multiple sources. It is possible that the transcripts aren't translated into proteins, yet if they are

one could envisage that the activity of the protein is inhibited by hypoxia. But most importantly, as already explained in the results, additional replicates should be performed in order to determine whether these tendencies validate themselves or not.

The only significant effect that hypoxia had on MCF-7 MS was on the initiation of MS formation. Hypoxia induced an increase in the number of MSs formed but only when the adherent cell line was pre-incubated for 48 h in hypoxic conditions and after that the cells were seeded in hypoxic MS forming conditions. Therefore, it could be of interest to study the metabolism of HA in these same circumstances. It would also be of interest to understand the mechanisms underlying the improved MS initiation during prolonged hypoxia. The results indicate that hypoxia may have a way of preparing the CSC present in the adherent population for subsequent survival in low oxygen and anchorage independent conditions. The gathered results point out that hypoxia probably doesn't increase the number of CSC in the adherent ("parental") culture, since no effect was visible in the hypoxic to normoxic conditions of culture. In order to further understand why the MS formation is increased in the strictly hypoxic conditions, future studies could explore the influence of ER- α and the formation of secondary spheres. Additionally one could also attempt to study the expression of stem cell markers, such as Notch, Nanog, Oct4 and CD44 shortly after seeding, in order to determine whether the different oxygen combinations induce different levels of expressions in the 4 conditions. Preferably this would be done one day after MS seeding in order to really understand how the MS is formed. Performing all of these studies on other cell lines including the MDA-MB-231 cell line would of course also be interesting. A minor limitation that accompanies studying the actual initiation of MS development is the fact that the cells are seeded at rather low densities. This means that there is not much material that can be collected at the start of MS formation. The preliminary results conducted by my colleague who examined the expression of one of the stem cell markers, CD44, by RT-qPCR in 2-day-old MSs show a 5-fold increase in expression. At 4 days the level of expression significantly decreases. This indicates that studying the stemness markers as early as possible after seeding is crucial. Studies carried out at later periods mean that the MS already have a lower proportion of CSC due to the fact that the stem cells will have undergone asymmetric divisions, giving rise to a more differentiated progeny.

As already evoked in the article published by Harrison *et al.*, for an optimal cancer treatment it will be extremely important to determine in what way the ER- α status can bequeath CSCs with increased self-renewal abilities or increased dedifferentiation of progenitor cells in hypoxic conditions. Based on this status, the treatment approach will have to be modified. If the CSCs in ER- α positive cell lines have amplified stemness abilities in hypoxic conditions, the anti-angiogenic drugs would not be the appropriate therapy. These treatment options could even be catastrophic for the patient as they would favour the CSC component of the tumour, which could favour cancer progression. These findings value the importance of treating patients in function of the particular cancer subtype their body is burdened with (Harrison *et al.*, 2012). The findings of this Master's Thesis may be preliminary but they do contribute to the fact that an ER- α positive cell line has increased CSC activity in hypoxic conditions. This is an important conclusion. The protocol and the conditions for MS formation we use are not identical to that of Harrison's model, but that difference in technical approaches may be considered as beneficial, as it shows that even under other circumstances the hypoxic effect of increasing CSC activity in MCF-7 cells can be observed.

MDA-MB-231

In the framework of this particular study the MDA-MB-231 cell line appears to have a much larger response to hypoxia. MS cultured in hypoxia can develop into larger formations over time than those of normoxic cultured MSs. The medium in which the MS are grown is of significantly lower pH than that of the normoxic spheres. And very interestingly the expression of HAS2 as well as the quantity of HA is increased in hypoxic conditions. One could be tempted to imagine that the increase in HAS2 expression in MDA-MB-231 MS could be linked to the pH of the medium. The normoxic and hypoxic pH profiles are similar between both cell lines, whereas the transcriptional expression of HAS2 is very different between the different cell lines. This indicates that the pH is probably not the reason for the increased expression of HAS2.

The larger size of the hypoxic MS could be explained by increased proliferative or self-renewal activity within the MS. This could be verified by analysing the abundance of Ki67, a proliferation marker. This is a plausible hypothesis as in the literature hypoxia has been associated with increased proliferative activities (Keith & Simon, 2007). As already mentioned in the results, if the increased size is due to altered cell-cell or cell-ECM interaction it could be a consequence of elevated CD44 expression or an elevated amount of HA synthesis which would considerably modify the ECM. The RT-qPCR analysis rules out the possibility that increased cell-cell interaction is due to a hypoxic induced elevation of CD44 expression. If the increase in MS size is due to increased survival of the cancer cells, this could be mediated through the expression of different CD44 splice forms without affecting the global level of CD44 expression. The expression of CD44v3 for example has been associated with increased tumour cell survival. Once HA has bound to its receptor, then Oct4, Sox2 and Nanog form a complex that can activate the production of miR-302. This small non-coding RNA can up-regulate survival proteins such as IAP.

Of course many other proteins could be involved in modifying the adhesion between cells and their ECM, for example CD99, α -integrins and collagen 5 α 1 (Harris, 2002). The study of any of these could possibly supply the required information to understand this increased MS size in hypoxia. But as they are not relevant to the metabolism of HA, those studies were not performed.

HAS2

The other element that could explain the increased MS size and the hypothesized favourable cell-cell interactions is an elevated level of HA synthesis. The RT-qPCR analyses reveal that there is in fact an increased expression of HAS2 in the hypoxic MDA-MB-231 MSs. This elevated expression of HAS2 after 7 days in hypoxia would support the theory that HA could have an impact on the size of the MS, as an elevated amount of HAS2 indicates that there could be a more abundant production of HA. As the expression of HAS2 is based on mRNA it would be good to confirm that this elevated HAS2 expression is also valid at the protein level. The HA dosage indicates that hypoxia does induce a higher production of HA in MSs although not dramatically. The cellular fraction of hypoxic MSs presents a significantly increased quantity of HA at developmental day 4, and a tendency to an increased abundance at day 7. The HA released into the medium also appears to be higher in the hypoxic MSs. These results are partly consistent: there is a slight inconsistency when considering at what point in development the increases of HA and HAS2 occur. As already mentioned the transcriptional expression of HAS2 shows a tendency to increase at day 4 but is only significant at day 7, not as one would have expected. Increased replicates for both assays

could elucidate this inconsistency. The increased production of HA in hypoxic MSs could explain increased cell-cell interactions allowing this particularly fragile structure to be more resistant to the manipulations necessary to analyse them. The interaction that can take place between the receptors present on the surface of tumour cells and the HA of the ECM can also mediate signalling pathways which could increase the survival of tumour cells and could also contribute to the increased size of these hypoxic MSs. One example of such an interaction is CD44-bound HA. This interaction can decrease apoptosis and increase tumour cell survival via the intermediate of PKC activated Nanog, which could contribute to the increased size of these MSs. This very same interaction can also stimulate ERBB2 signalling which inhibits the pro-apoptotic protein Bad (Bourguignon et al., 2009; Toole, 2004).

The expressions of different HASs, especially HAS2, are known to be altered by PDGF-BB, TGF, glucocorticoids and lactate. Investigating what induces the increase in HAS2 expression should be a step performed in future studies. The up-regulation could be a direct response to hypoxia via one of the HIF proteins but the literature doesn't evoke the presence of HREs in the promoter regions of this HA synthesising protein. It is also possible that the expression of HAS2 is indirectly affected by hypoxia. The response could be mediated by the altered metabolism that can occur in low oxygen conditions. The presence of lactate-sensitive elements in the promoter regions of HAS2 haven't been documented either. As the MDA-MB-231 MS medium undergoes significant decrease of the pH under hypoxic conditions, one could believe that the cells composing the MS may be in acidosis, or at least have a decreased intracellular pH. But due to the fact that the pH measurements of MDA-MB-231 and MCF-7 MS are similar yet the expression of the HAS enzymes does not follow this pH profile it is improbable that it is the pH that induces the difference in HAS2 expression. This does not exclude the fact that there may be a higher abundance of lactate within the cells which may affect the expression of HAS2. TGF- β is a direct target of HIF-1 α , thus the elevation of HAS2 in response to hypoxia may be an effect of TGF- β . In fibroblasts glucocorticoids have been shown to decrease the transcription of the HAS2 gene, meaning that glucocorticoids negatively regulate HAS2. Various non-cancer-related studies have uncovered a link between hypoxia and glucocorticoids. Hypoxia can increase the levels of glucocorticoid receptors, enhancing the sensitivity towards these steroid hormones. The fact that hypoxia has been seen to increase the sensitivity to these stress response hormones elicits that it's not the glucocorticoids that induce the up-regulation of HAS2 (Bernert, Porsch, & Heldin, 2011; Kodama et al., 2003; Leonard, Godson, Brady, & Taylor, 2013; W. Zhang, Watson, Liu, Williams, & Werth, 2000). To better understand this hypoxic response it would be interesting to further investigate how the up-regulation of HAS2 expression is mediated.

The importance of HAS2 as a crucial factor for metastatic progression is still a relatively new concept that should continue to be explored. Studies performed in 2005 are amongst the first to tie elevated HAS2 activity to breast cancer progression (Udabage et al., 2005). In 2011 an article evoked that HAS2 mediates malignant progression by suppressing tissue metalloproteases-1 inhibitor (Bernert et al., 2011). This suppression allows the MMPs to accomplish the degradation of the ECM which is a necessary step for cellular migratory activities, a characteristic of metastatic cells. One year later an article appeared linking the HAS2 up-regulation to bone metastasis by breast cancer stem cells (Okuda et al., 2012). The high expression of this protein enabled the breast CSCs to interact with tumour associated macrophages, which in return secreted a growth factor, PDGF-BB, which stimulates stemness pathways such as self-renewal. The interaction between TAMs and CSCs with an elevated HAS2 expression is mediated via the abundant presence of CD44 on the TAMs and the increased production of HA by the CSCs. The increased production of HA by these CSCs is a consequence of the elevated expression level of HAS2. In the CSCs this enhanced expression of the HA synthesizing protein also promotes the abilities to adhere and to migrate.

The novel finding that hypoxia increases the expression of the HAS2 gene in MDA-MB-231 offers some new insight into how hypoxia could contribute to the malignant progression of CSCs. As already mentioned further examination must be completed in order to better understand how hypoxia can contribute to this increase in HAS2 expression in this CSC model. If this adaptation does in fact contribute to malignant progression and is a phenomenon present in *in vivo* tumours it could be possible to use the increased expression to the cancer patient's advantage. Treatment with 4-methylumbelliferone could be an option, as this molecule depletes the substrate necessary for HA synthesis and down-regulates the mRNA for HAS enzymes (Kultti et al., 2009). Okuda *et al.* have already established that treatment with this small molecule impairs HAS2 mediated *in vivo* metastasis (Okuda et al., 2012). It must also be determined whether this gripping response is cell line specific, whether it's dependent on the expression of a particular receptor such as ER- α and whether it is a common occurrence in hypoxic breast CSCs. The MCF-7 cell line doesn't have the same response, evoking that it probably isn't a global breast CSC response to hypoxia. Evidently the metastatic properties, enhanced adherence and migratory abilities, that an elevated HAS2 expression could potentially confer on CSCs must also be tested in this particular model, which could back the results published by Okuda *et al.*

Hypoxic effects have been seen to outlast the actual duration of hypoxia. This can be caused by epigenetic alterations. Through these alterations the genes up- or down-regulated during hypoxia can be maintained in the state of activity that the hypoxic surroundings induced. Such epigenetic alteration in response to a certain stress hasn't solely been reported in hypoxic breast CSC: a study on human aortic endothelial cells incubated in hyperglycaemic conditions has also revealed that the stress induced response outlasts the duration of the stress (El-Osta et al., 2008). This is particularly interesting, as hypoxia is not always chronic in tumour masses. The oxygen tension within solid tumours can develop over time and is heterogeneous throughout the mass; this means that the cancer cells may succumb to varying concentrations of oxygen (Vaupel et al., 2007). This "memory" to stress responses such as hypoxia is quite dismaying as a brief encounter with the stressful environment is enough to change the cellular mechanisms and offer a cell lasting abilities that could contribute to cancer progression. This is only one of the remarkable ways tumour cells are able to overcome and take advantage of the biology of the entire organism.

Brief summary

In summary, our findings indicate that hypoxia clearly has an effect on both cell lines grown in anchorage independent conditions. This said, the oxygen concentration has very different effects in these cell lines: it is only in MDA-MB-231 MSs that the metabolism of HA is altered. The MCF-7 cells see an increase in the number of MSs formed under strictly hypoxic conditions, when both the pre-treatment and the MS seeding are carried out in low oxygen tensions, indicating that hypoxia may prepare the CSCs to survive in low oxygen conditions (epigenetic phenomenon?). The fact that MDA-MB-231 cells see an increase in HA quantities and in HAS2 expression at the RNA level still needs to be further elaborated, but this preliminary result indicates that this may be a novel pathway to how hypoxia contributes to the malignant progression of cancer.

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